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Clinical, molecular genetic and functional studies on inherited human cataracts

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Abstract

Cataracts, the commonest cause of blindness in the world, may be broadly divided into adult onset and childhood onset. Some childhood cataracts are present from birth (congenital cataracts). About one third of congenital cataracts occurring as isolated abnormalities, and not part of a syndrome, are inherited. Full clinical examination was performed on individuals from families with inherited cataracts. DNA was extracted from peripheral blood samples. Linkage analysis was performed in three large families.

Key individuals were examined from two of these families with autosomal dominant congenital cataract (ADCC). In one of the families, cataract was linked and linkage refined to a region encompassing the transcription factor gene, *PITX3*, known to be associated with cataracts and anterior segement mesenchymal dysgenesis (ASMD). Both families were found to have the same mutation in this gene, which segregated with disease and was absent in control individuals. The phenotype in these families, posterior polar cataract with or without ASMD, is different from that previously reported for this mutation and shows both inter- and intra-familial variability.

Autosomal dominant (AD) zonular pulverulent cataract in a third large family was linked to the connexin 46 gene (*CX46*), known to be associated with cataracts. Sequence analysis identified a novel mutation in this gene which segregated with disease in the family and was absent in control individuals. Wild type and mutant constructs of *CX46* were prepared and expressed in human HeLa cells. These

studies demonstrated that the mutation does not affect trafficking of the protein to the cell membrane.

Individuals from twenty-one further families with inherited cataract were examined clinically. Their DNA samples were added to the existing panel for candidate gene screening.

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Publications and Presentations arising from this work

Addison PKF, Berry V, Holden KR, Espinal D, Rivera B, Su H, Srivastava AK, Bhattacharya SS. A novel mutation in the connexin 46 gene (*GJA3*) causes autosomal dominant zonular pulverulent cataract in a Hispanic family (in submission).

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Oral Presentations

Addison PKF. Connexins and the lens. Ophthalmology-Genetics Meeting, Institute of Child Health, London, 2005

Addison PKF, Berry V, Ionides ACW, Francis PJ, Bhattacharya SS, Moore AT. The clinical consequences of a recurrent mutation in *PITX3*. European Paediatric Ophthalmology Society Annual Meeting, Manchester, 2004

Poster Presentation

Addison PKF, Berry V, Holden K, Rivera B, Su H, Srivastava AK, Bhattacharya SS. A novel locus for human polymorphic congenital cataract. ARVO, Florida, USA, 2004

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Statement of originality

The work presented in this thesis submitted for the degree of Doctor of Medicine is my own composition and except where otherwise stated the data presented herein is my own original work.

Peter K. F. Addison

Dedication

To Emma...

1.0 Introduction

1.1 Cataract as a Disease

Cataract, opacification of the crystalline lens of the eye, is the commonest cause of blindness in the world. The World Health Organisation estimates that 45 million people in the world are blind, 19 million of them as a result of cataract.[1] Cataracts may be broadly divided into adult onset and childhood onset (either congenital or infantile). Childhood cataract is both phenotypically and genotypically heterogeneous. Congenital cataract is defined as cataract which is present from birth and is responsible for approximately one-tenth of worldwide childhood blindness.[2] Childhood cataracts account for about 10-20% of childhood blindness in developing countries[3] and about 4% of adult blindness in industrialised countries.[4] According to the US collaborative perinatal project,[5] childhood cataract has a prevalence of 13.6 cases per 10,000 live births. The incidence of congenital cataract in the UK and Australia is between 2.2 and 2.49 per 10000 live births.[6;7]

The visual outcome for congenital cataract depends on whether the cataract is total or not at birth, whether the cataract is progressive or stationary and, in the case of severe cataract, the age at surgery. Total congenital cataract requires early cataract surgery and visual rehabilitation. This contrasts with partial progressive cataract, for which surgery can often be delayed until later childhood with good visual outcome.[8] Partial stationary cataract may not require surgery and has the best prognosis of all.

Childhood cataracts may be inherited or non-inherited. Intrauterine infections (rubella, varicella, toxoplasmosis), trauma and juvenile ocular inflammatory

disease are the commoner causes of non-inherited cataract.[9] Inherited cataract may occur as an isolated abnormality, as part of a more complex ocular developmental abnormality or as part of a systemic syndrome. About one third of isolated congenital cataracts are inherited,[10] the commonest mode of inheritance being autosomal dominant.[7;10] Autosomal recessive (AR) and X-linked recessive modes of inheritance are also seen but are uncommon. The emphasis of this thesis is on investigating and adding to existing knowledge and understanding of isolated ADCC.

1.2 Normal Lens Biology

1.2.1 Embryology

1.2.1.1 Lens vesicle formation

In humans, eye development begins around day 22 of gestation, when the embryo is approximately 2mm in length.[11] Two hollow diverticulae, optic vesicles, are formed from neural ectoderm of the prosencephalon. The lens placode is recognisable on day 27 as a localised thickening of the surface ectoderm anterior to the optic vesicles. The single-layered spheroidal optic vesicle undergoes a critical transformation by means of active invagination to become a goblet-shaped optic cup. The lens placode invaginates in turn via a combination of differential growth and buckling to form the lens vesicle. By day 29, invagination of the lens placode is almost complete. A small lens pit can be identified just before the surface ectoderm seals over. As the vesicle, surrounded by its basal lamina, detaches from the surface ectoderm, it sinks into the underlying rim of the optic cup. By the start of day 36, the lens vesicle separates from the surface ectoderm.

The lens epithelial cells enclose the lens cavity and are surrounded externally by a basal lamina which will form the future lens capsule. Following separation of the lens vesicle from the surface ectoderm, this layer regenerates to form the future corneal epithelium.

1.2.1.2 Primary lens fibres

In response to an inductive signal from developing neural retina, the posterior cells of the lens vesicle elongate to form the primary lens fibres and begin synthesis of a new group of intracytoplasmic proteins known as crystallins. The base of each elongating lens cell remains anchored to the basal lamina posteriorly and their apices grow towards the anterior lens epithelium, thereby obliterating the lens cavity. The anterior cells of the lens vesicle remain as a single layer of cuboidal epithelial cells. The lens vesicle becomes a solid ball of cells by the end of the fourth week and is known as the embryonic lens nucleus.

1.2.1.3 Secondary lens fibres

Subsequent lens fibres arise from anterior lens epithelial cells that migrate equatorially and undergo a single mitotic division following which further division does not occur. They are known as secondary lens fibres. The main function of the lens epithelium is to produce lens fibres and synthesise lens proteins. The newly differentiated fibre cells are deposited in concentric lamellae, resembling an onion skin, in a strictly ordered manner and continue to be formed throughout life. Superficially located lens fibres are rich in ribosomes, polysomes, and rough endoplasmic reticulum. They actively synthesise lens crystallins,

amongst other proteins. Maturation of the lens fibres is associated with loss of nuclei and all intracellular organelles. This has important implications. Mature lens fibres are not capable of fulfilling their metabolic cellular requirements. It is for this reason that intercellular communication between immature but metabolically active lens epithelial cells and mature but metabolically inactive lens fibres is essential. The lens functions as a syncitium, which will be discussed in section 1.2.2.3.

The tips of the secondary fibres extend around the primary fibres and meet at the Y-shaped anterior and posterior lens sutures.[12] Every subsequent generation of fibres throughout life is added superficial to the previous layer. Early in embryonic development, the lens is nearly spherical. However, as secondary fibres are added at the equator, the lens becomes more ellipsoid, a trend that continues throughout life. Basal lamina material is continually deposited by the lens epithelium on its external aspect and encases the lens in a membranous non-cellular envelope, the lens capsule. During embryonic and foetal development, the lens receives nourishment via an intricate vascular net, the tunica vasculosa lentis that completely encompasses the lens by approximately nine weeks.

1.2.2 Anatomy, biochemistry and physiology

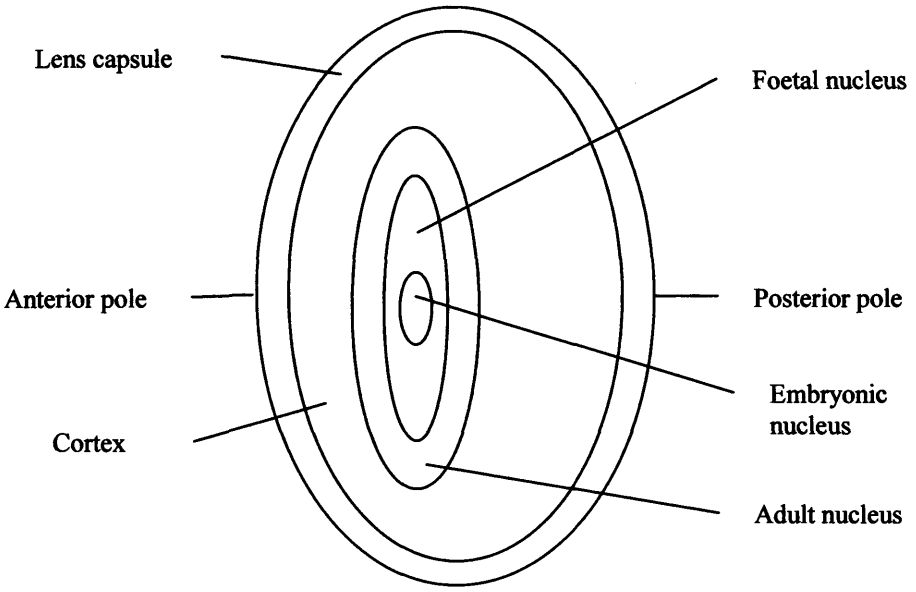
1.2.2.1 Structure and Function

The lens is made up of three parts:

1. an elastic capsule
2. an anterior lens epithelium
3. the lens fibres

The lens capsule serves as a diffusion barrier. It is freely permeable to low molecular weight compounds but restricts the movement of large colloidal particles. The function of the lens epithelium is mainly twofold. The cells located at the equator are actively dividing, differentiating into lens fibres and synthesising proteins, including crystallins. The remaining, more centrally placed cells are involved in the transport of substances from the aqueous humour to the lens interior, and in the secretion of capsular material. As a result of the continuous production of lens fibres from the embryonic stage and the progressive internalising of the fibres, the earliest fibre mass in the centre of the lens is referred to as the embryonic nucleus. This is surrounded by the foetal nucleus with its Y-shaped sutures. Those fibres formed after birth constitute the earliest part of the fibre mass known as the adult nucleus. The area surrounding the adult nucleus, containing the recently formed nucleated fibres is called the lens cortex. The centre points on the anterior and posterior surfaces of the lens are known as the anterior and posterior poles. A schematic diagram of the lens showing all these zones is shown in Figure 1.

Figure 1 Schematic diagram of a human lens in sagittal section



Lens fibres are hexagonal in cross section and are tightly packed with very little intercellular space. They are held together by the interlocking of their adjacent plasma membranes. The membranes contain numerous gap junctions. The close relationship that exists between the lens epithelial cells and the lens fibres, as seen by the interdigitation of the plasma membranes and the existence of numerous gap junctions, makes the lens a syncitium. From a physiological point of view, the lens acts as a single cell. Its extensive intercellular gap junction communication system maintains tissue homeostasis and hence transparency.[13]

1.2.2.2 Lens size and shape

At birth, the crystalline lens is a transparent biconvex structure with an equatorial diameter of 6.5 mm and an anterior-posterior depth of 3.5 mm between its poles. The continual addition of lens fibres increases the equatorial diameter to 10 mm and the anterior-posterior depth to 4 mm by adulthood. The lens continues to gradually enlarge throughout life.

1.2.2.3 Lens homeostasis

During the fourth and fifth months of gestation, the tunica vasculosa lentis gradually atrophies. Following complete regression of the vascular system, the lens becomes an avascular organ with no lymphatic drainage. The lens fibres are now dependent on anterior epithelial cells to maintain homeostasis. These cells remove waste products and exchange them with nutrients from the aqueous humour through the semi-permeable capsule membrane.

The lens behaves as a syncitium in which potassium ions (K^+) are transported into the lens and sodium ions (Na^+) are transported out via Na^+/K^+ ATPase present in the lens epithelium. The lens also contains specific glucose transporters and transporter molecules for ascorbate, which ensure adequate metabolism and minimise free radical damage. Glucose is derived from the aqueous, as are amino acids which are actively transported across the epithelium.

1.2.2.4 Lens cell membranes

Gap junction communication takes place between the aqueous humour and the anterior lens epithelial cells and between lens fibre cells. At the junction between the epithelium and the fibre cell, the main transport mechanism is rapid endocytosis via coated vesicles. Major intrinsic protein (MIP) is the principal protein constituent (50%) of fibre cell membranes, but it is absent from epithelial cells. MIP forms water channels. Membrane lipids are only produced by peripheral lens cells. There is no turnover of lipid in the membranes of mature fibre cells.

1.2.2.5 Lens metabolism

The lens has most of the biochemical pathways found in other tissues but all processes are not present in all cells because of the requirements for both transparency and a high refractive index. A high content of coloured molecules such as cytochromes and flavoproteins would absorb too much light, and if all cells contained a full complement of mitochondria and other organelles such as

the cell nucleus, too much light would be scattered. For similar reasons the lens is avascular.

The lack of organelles in mature lens fibres has two biochemical consequences. Firstly, the majority of the lens must obtain its energy from glycolysis, not from oxidative phosphorylation. Secondly, there is no synthesis of protein in the centre of the lens. Glucose from the aqueous humour is the main source of energy for lens metabolism. Glucose enters lens epithelial cells via insulin-dependent glucose transporters located in the plasma membrane. Approximately 90% of lenticular glucose is metabolised via the glycolytic pathway. As the lens fibre cells have no mitochondria, only epithelial cells are able to use oxidative phosphorylation for further ATP production. Under physiological conditions most glucose is metabolised via glycolysis to lactate, with the remaining 10% entering the pentose phosphate pathway for the synthesis of pentoses and NADPH. Sorbitol has been shown to accumulate in diabetic rat lenses, and this reaction was thought to be secondary to the activity of aldose reductase. In fact, although the enzyme exists in the lens, recent studies suggest that glucose may be an incidental substrate of aldose reductase and that sorbitol accumulation does not result in cataractogenesis.[14] Cataract formation in diabetes is primarily caused by glycosylation of lens proteins, with the subsequent disruption of the Na^+/K^+ ATPase pump, and not by sorbitol accumulation.

Glutathione is found in high concentrations in the lens.[15] Mostly (93%) it is found in its reduced form (GSH), and is maintained thus by the NADPH produced by the hexosemonophosphate shunt. A reduction of lens GSH is a consistent finding

in age-related and all tested experimental cataracts. Glutathione peroxidase is involved in the detoxification of hydrogen peroxide, some of which is formed from O_2^- radicals.

1.2.2.6 Function of the lens

The purpose of the lens is to refract light rays onto the retina. The dioptric power of the entire eye is about 58 dioptres, with the cornea responsible for most of this refractive ability. The lens contributes only about 15 dioptres to the total power. The importance of the lens is that it can change its dioptric power, allowing distant and near objects to be focused onto the retina. The high refractive index of the lens is due to the crystallins. The transparency of the lens is due to the shape, arrangement, internal structure, and biochemistry of the lens cells and lens fibres.

1.2.2.7 Lens transparency

The transparency of the lens is largely the result of the structured packaging of macromolecular components within the lens fibres and the small differences in refractive index between light scattering components. The regular arrangement of lens fibres and the absence of tissues such as blood vessels and nerves also minimise light scatter. Lens transparency is a crucial trade-off between the concentration of the macromolecules and their hydration. Disruption of normal levels of hydration can lead to opacity. The single-celled epithelium of the lens and its capsule do not scatter or reflect light, essentially because the combined refractive index is the same as that of the aqueous humour.

Light transmission by the lens is reduced when the ordered packing of the lens crystallins is disturbed. This can be induced in many ways, such as increased water accumulation within the lens, formation of high molecular weight lens protein aggregates, and vacuole formation within the lens fibres with age. Since the lens is designed for the transmission of light, it responds to any insult that disturbs normal development or metabolism, by opacification, even if the insult is only temporary.

1.3 Cataract phenotypes

The morphology of congenital cataract reflects a combination of the timing and nature of the cause, the anatomy of the lens, its pre- and post-natal development, and changes that take place with time.[16] The morphology of congenital cataract is largely determined by the anatomy of the lens and the timing of the insult in embryogenesis. Morphology may variably affect prognosis, give a clue to the age of onset and, in an isolated case, sometimes suggest heritability. Certain phenotypes (lamellar, pulverulent, polymorphic, coralliform and cortical) seem to have good visual prognosis.[8]

Many classification systems have been proposed to categorise human inherited cataract morphology.[9] This research focuses on isolated ADCC. Ten distinct clinical entities have so far been described for this subset of inherited cataracts in separate studies.[17;18] These phenotypes together represent a clinical classification system for this particular disorder. I have used this classification system which incorporates both the position of the opacity within the lens (Figure 1) and its

appearance on slit-lamp examination. The ten separate phenotypes are described in this section, along with sutural cataract which, in AD disease, is only ever present in conjunction with at least one of the other phenotypes.

1.3.1 Anterior polar cataract (APC)

Anterior polar cataracts are situated at the anterior pole of the lens. They are usually symmetrical and discrete lesions. Anterior polar cataracts vary from single dot-like opacities to larger pyramidal-shaped opacities.[18;19]

1.3.2 Posterior polar cataract (PPC)

Posterior polar cataracts are located at the posterior pole of the lens. They are commonly symmetrical. PPC may be divided into stationary[20;21] and progressive types.[22;23] The progressive subtype spreads from the posterior pole over time. It is associated with a higher incidence and an earlier age of surgery.[23;24] In PPC, opacification is close to the optically crucial, nodal point of the eye. Therefore, vision is commonly reduced.[25]

1.3.3 Nuclear cataract

Early onset nuclear cataracts are located in the embryonic and foetal nucleus of the lens. They vary widely in appearance and the need for surgery depends on the type of nuclear cataract. Confluent nuclear opacification often requires early surgery. On the other hand, blue dot nuclear cataract may not require surgery. Nuclear cataracts are usually static. Attempts to differentiate between embryonic

and foetal nuclear cataracts were made in the past but are extremely difficult to make clinically. It is much easier to define these cataracts as nuclear and then subclassify them according to slit lamp appearance.

1.3.4 Lamellar

During normal lens growth, lamellae are formed from the concentric deposition of newly differentiated secondary lens fibres around the embryonic and foetal nucleus. Lamellar cataracts involve one or more lamellae or layers of the lens, as a shell of opacity, sandwiched between clear nucleus and cortex.[9;26] Lamellar cataract, sometimes referred to as zonular cataract, represents several generations of secondary lens fibres, which have become opacified in response to an insult when these fibres were at their most metabolically active. The opacification of discrete lamellae and the normal production of later lamellae suggest that the disruption to lens development is a transient event in this type of cataract. The majority of families develop lamellar cataract before birth. The density of the cataract varies significantly between individuals. The opacity may be so dense as to render the entire central region of the lens completely opaque, or so translucent that vision is hardly affected at all.

Typically, lamellar cataracts are bilateral but slightly asymmetrical, sometimes with different degrees of opacification in different meridians. They are composed of minute white dots in one or more layers of the lens, not involving the embryonic nucleus. They are usually sharply demarcated from a clear cortex outside them. They are often incomplete, and they may have projections from their outer edges known as riders. The visual prognosis, especially in partial

cataract, is probably better than in many other morphological types.[27] Many cases can be managed conservatively and surgery in infancy is rarely necessary. However, there is often a marked intrafamilial variability[28] and some lamellar cataracts have a poor visual outcome. This occurs if the opacity starts earlier during lens development, if the cataracts are denser and if they involve the centre of the lens. In such cases, profound amblyopia is common.

1.3.5 Pulverulent

Pulverulent cataracts have a pulverised (powdery) appearance to the opacification. Pulverulent cataracts have been reported in both AD[29] and AR[30] disease. Pulverulent opacities may be located throughout the lens or in a particular region. Nuclear pulverulent cataracts[31-33] are confined to the nuclear region of the lens and so are also a subtype of nuclear cataracts. "Zonular pulverulent cataract" is a term which has been used to describe pulverulent cataracts which involve the nucleus minimally but markedly affect lamellar regions beyond it.[34-39] Pedigrees affected by pulverulent cataract may show a wide intrafamilial variation in the phenotype.[18]

1.3.6 Coralliform

This is a rare form of congenital cataract. It is characterised by finger-like projections which resemble sea coral extending from the nucleus into the cortex.[18] The visual impact is variable but cataract extraction is usually required in the early years of life. Coralliform cataract may well overlap with a similar rare phenotype, aculeiform or "needle-shaped" cataract.[40]

1.3.7 Cerulean

Cerulean cataract is a distinctive type of cataract, both in terms of appearance and prognosis. It has not been observed in a neonate so may be a form of infantile rather than congenital cataract. It has been reported in childhood and progresses throughout early life. Discrete pinhead shaped blue and white opacities are distributed throughout the lens, becoming more numerous in the cortex where they may form large cuneiform (wedge-like) shapes in the mid-periphery.[18] Within a pedigree, this phenotype is consistent in its distribution but variable in its severity. Visual acuity is usually well preserved. Often surgery is not required until adulthood and it is usually associated with a good outcome.[41]

1.3.8 Cortical

This form of inherited cataract is rare. Opacification affects a sector of the lens in the outer cortex adjacent to the capsule.[18] This represents a late insult since there is opacification in the more newly formed secondary lens fibres, which lie in the equatorial region of this zone. The other regions of the lens, including the visual axis, are clear. Therefore, an affected individual's visual acuities are not reduced and the prognosis is excellent.

1.3.9 Polymorphic

Some cataract phenotypes show considerable variation in morphology even within the same family. The polymorphic cataract phenotype was first described in three

families with wide variations in grape-like lens opacities that spared the nucleus.[42] The distribution of the opacities varied from the superficial to the deep layers of the cortex. The term polymorphic has since been used to characterise the cataract in another unrelated family with a similar distribution of opacities in association with asymmetric polar opacification.[17]

1.3.10 Total

AD total congenital cataract has recently been reported in an Australian family.[43] Early cataract surgery is essential to limit amblyopia due to total lens opacification.

1.3.11 Sutural

This is not really a separate phenotype since it is rarely inherited without other forms of morphological change within the lens. In fact, there are currently no reports of isolated AD sutural cataract. The cataract consists of prominent opacification of the anterior and posterior sutures. Sutural opacities have been described in association with lamellar,[44] pulverulent[29] and nuclear[45] cataracts. Isolated sutural opacities, although not reported in any AD pedigree, may be seen in female carriers of X-linked cataract, particularly Nance-Horan syndrome.[46]

1.4 Genetic concepts

1.4.1 The human genome

The human genome has now been completely sequenced. A large number of disease loci have been mapped. In addition, genetic markers throughout the genome are available to help in mapping diseases to chromosomal loci. A genetic marker is any polymorphic Mendelian character that can be used to follow a chromosomal segment through a pedigree.[47] Microsatellite markers are di-, tri-, and tetra-nucleotide repeats which are distributed throughout the genome. There are potentially more than 10^5 microsatellite loci. Each has many alleles and they are highly informative. By using informative markers in large families with ADCC, linkage can be established and the chromosomal locus of the causative gene identified.

1.4.2 Linkage

The principles of linkage are fundamental to understanding the molecular genetics of any inherited disease, particularly ADCC. The following explanation should aid this understanding.

If one flips a coin, just by chance alone, there is a 50% chance that it will land heads and a 50% chance it will land tails. If one flips a coin ten times, just by chance alone, it will land heads five times and it will land tails five times. If it lands heads more than five times or less than five times, the further away from five times this happens, the less likely that this has happened by chance alone, and

the more likely that something has caused this outcome number to be skewed one way or the other.

Similarly, if one investigates a family in which a disease is inherited and looks at a marker at a specific place on a chromosome, there is a 50% chance that the marker and the disease will segregate together by chance alone, i.e. that no crossover will intervene. A crossover, that is to say, exchange of genetic material between a pair of chromosomes, separates two points on a chromosome that are far apart. If the marker and the disease segregate together in more than 50% of the informative meioses, for example in more than five out of ten children in a large family, then the more times they segregate together, the more likely that the marker and the disease phenotype are located close together on the chromosome. This observation is compatible with linkage. The closer two points are on a chromosome physically, the more likely they will be inherited together. The more the number of informative meioses in which the two points segregate together, the closer together the two points are. If one looks at enough informative meioses, i.e. a large enough family, soon a point will come where the chances of the two points segregating together by chance alone is so small that it is not happening by chance. Rather, the two points are physically extremely close. This observation is compatible with linkage.

The probability of flipping a coin and getting heads ten times in a row is worked out as: ½ x ½ x ½ x ½ x ½ x ½ x ½ x ½ x ½ x ½ = 1/1024. This is the odds ratio. Only once in 1024 times will one flip a coin and get ten heads in a row by chance alone. 1023 times one will get at least one tails. Now, the LOD score gives the

logarithm of the odds ratio. This is a quantitative measurement of linkage. For example, if the odds ratio was one thousand, there would be a one in a thousand probability of a particular event occurring by chance alone. The LOD score in this case would be three, the logarithm of one thousand. A LOD score of three or more indicates that the odds of linkage being present are one thousand to one in favour of linkage. 999 times out of one thousand this result is happening because there is linkage. Only once in one thousand times is it happening by chance alone.

Now, a marker can be relatively close to a disease locus. In this case, the percentage of meioses in which the two points are linked is higher than by chance alone, but they may not segregate together as linked loci in all the meioses. The closeness of the marker to the disease is reflected by the theta value (θ), the recombination frequency. For instance, if a significant maximum LOD score is obtained at a theta value of 0.01, this is saying that 1centiMorgan (cM) away from the marker, the disease gene is likely to be found. Two loci that show 1% recombination between them are defined as being 1cM apart on a genetic map.

1.4.3 Principal ways of finding genes in families with ADCC

There are two main methods of finding these genes. If the family with ADCC is large enough to provide a sufficient number of informative meioses, linkage can be established. Linkage is established when the disease locus and the genetic marker used are sufficiently close together on the chromosome. Using other nearby genetic markers, the points on the chromosome where the upper and lower (flanking) crossovers are found can be established. This leads to a region of linkage being refined. Within this region, candidate genes can be sequenced

looking for a mutation which segregates with cataract in the family. Additional mutations in other families confirm the involvement of that gene in cataractogenesis.

If the family is too small for linkage to be statistically possible and if the phenotype is highly suggestive of a known cataract gene, then that gene can be sequenced directly in an affected individual to look for a mutation. A true cataract-causing mutation should segregate with cataract in the family and be absent in a large control panel of individuals without cataract.

1.4.4 Cataract panel

New candidate genes can be screened across a panel of individuals from unrelated families affected with inherited congenital cataract. From each family, an affected individual is screened for mutations in a gene. If a mutation is found and is not present in an unaffected individual from the same family, the rest of the individuals in that family are screened. If the mutation segregates with cataract in the family, its presence is tested for in a large control panel of individuals without cataract to see if it is a true candidate gene. More than one mutation in different individuals from the panel implicates the gene in cataract.

1.4.5 Importance of accurate phenotyping

It is clear from the above section on linkage that inaccurate phenotyping, in particular describing an individual as affected when in actual fact they are unaffected and vice versa, can have serious effects on establishing linkage in a

family. In congenital cataract, it is extremely important to confirm a history of cataract from birth. Phenocopies, such as in age related cataract, if not recognised as such, could result in correct linkage not being found.

1.5 Cataract genetics

Currently fifteen genes are known to cause isolated inherited cataracts. Fourteen cause AD cataract and one causes AR cataract.[48] There are at least nine additional loci associated with cataract at which the causative genes have not yet been identified. Six of these are associated with AD cataract and three are associated with AR cataract.[48] In this section, the genes and loci reported to be associated with ADCC will be described.

1.5.1 Genes associated with ADCC

Mutations in genes encoding four major classes of proteins have been causally linked with ADCC. These classes of proteins are:

1. Crystallins, the predominant structural proteins in lens fibres
2. Lens fibre membrane proteins
3. Transcription factors important in lens development
4. Lens fibre cytoskeletal proteins

1.5.1.1 Crystallins

Crystallins are ubiquitous proteins found in many tissues where they serve multiple functions. Crystallins make up 90% of the water-soluble proteins of the lens. Three types of crystallins have been identified in mammals: α, β and γ, mainly differentiated on the basis of molecular weight. α-crystallins are the largest

lenticular proteins and consist of 30-40 subunits, which are of two types, αA and αB. These subunits undergo extensive enzymatic and non-enzymatic post-translational modification. As in all crystallins, the basic structure is a β-sheet. In addition to their role as structural lens proteins, α-crystallins have a role as molecular chaperones guiding the correct packing of other crystallins and folding of the cytoskeleton.[49] This phenomenon of multiple uses of a protein encoded by a single gene, termed "gene sharing", seems likely to be widespread in the lens, cornea and other ocular tissues.[50] β-crystallins are aggregates of polypeptides (23-35kDa) whereas γ-crystallins are monomers of 20kDa. There are acidic (A) and basic (B) β-crystallins.

During lens embryogenesis, an inductive signal from developing neural retina causes the posterior cells of the lens vesicle to elongate. They form the primary lens fibres and begin synthesising a new group of intracytoplasmic proteins, crystallins. Secondary lens fibres arise from anterior lens epithelial cells that migrate equatorially and undergo a single mitotic division following which further division does not occur. Superficially located secondary lens fibres are rich in ribosomes, polysomes, and rough endoplasmic reticulum. They also synthesise lens crystallins. As secondary lens fibres mature, they lose their nuclei and all intracellular organelles. Therefore, crystallin synthesis ceases. β- and γ- crystallins fold into four "Greek key motifs"[51] that facilitate tight packing of the molecules, thereby minimising light scatter. In contrast, α-crystallin, the most abundant lens crystallin, assumes a globular conformation that may be related to its role as a molecular chaperone.[52] The purpose of the lens is to refract light rays onto the retina. The high refractive index of the lens is due to the crystallins. The

structured packaging of crystallins in lens fibres and their solubility state are critical factors in maintaining lens transparency. Unsurprisingly, mutations in crystallins have been reported to cause ADCC.

1.5.1.1.1 α-crystallins

α-crystallins are of two types, αA and αB. A missense mutation, R116C, in the gene encoding αA crystallin (*CRYAA*) has been reported to cause nuclear cataracts.[53] In addition to cataract, five out of ten affected individuals in the family also had microcornea. This suggests that α-crystallins may play a role in the normal development of the anterior segment. However, this may be non-specific since any disruption to the normal early development of the lens may lead to consequences such as microcornea or microphthalmos.[54] Two mutations in *CRYAB*, the gene encoding αB crystallin, have been reported in association with ADCC. A deletion mutation, 450delA, has been reported to segregate with PPC.[20] The mutation resulted in a frameshift in codon 150 and produced an aberrant protein consisting of 184 residues. A missense mutation, R120G, has been reported in a family with cataract (no phenotype described in the paper) and desmin-related myopathy.[55] Presumably, the myopathy was caused by failure of the normal αB crystallin chaperone activity. In an elegant study, a mammalian two-hybrid system was used to investigate the effects of the R116C *CRYAA* and R120G *CRYAB* mutants on interactions between these proteins and other major lens constituents *in vitro*. The findings support the hypothesis that the functional effects of the mutations are to reduce protein solubility, increasing the likelihood of precipitation, seen clinically as cataract.[56]

1.5.1.1.2 β- and γ-crystallins

The β- and γ-crystallin polypeptides are recognised as members of a related β/γ-crystallin superfamily. The β-crystallin family consists of four acidic (A) and three basic (B) forms. γ-crystallins are also subdivided into a number of types. Of the γ-crystallins, only γC and γD, encoded by *CRYGC* and *CRYGD* respectively, are highly expressed in the human lens.

A number of mutations in β-crystallin genes associated with AD cataract have been reported. A nonsense mutation, Q155X, in the *CRYBB2* gene has been associated with cerulean cataract,[57] cerulean and sutural cataract[45] and nuclear pulverulent cataract[58] in three unrelated families. The variable phenotype with an identical mutation suggests that other modifier genes or, perhaps less likely, environmental factors may influence the phenotype. Two different splice site mutations in *CRYBA1* have been reported in unrelated families with pulverulent cataract[29;44] A *CRYBB1* nonsense mutation, G220X, has been implicated in a family with pulverulent cataract.[59]

A number of mutations in γ-crystallin genes have been reported in association with ADCC. A missense mutation, T5P, in *CRYGC* has been reported in association with pulverulent cataract.[60] A five base pair (bp) duplication mutation in *CRYGC* has been reported in a family with zonular pulverulent cataract.[61] A missense mutation, R168W, in *CRYGC* has been associated with lamellar cataract.[62] Several mutations in *CRYGD* have been reported in association with cerulean (P23T),[63] aculeiform (R58H),[60] nuclear (W156X)[62] and lamellar

(P23T)[62] cataract. A spontaneous heterozygous missense mutation in *CRYGD*, R36S, has also been detected in a boy with congenital prismatic cataracts.[64] Functional studies have shown that both the R58H and the R36S mutations reduce the solubility of CRYGD proteins rendering them prone to crystallisation.[64;65] This provides an explanation as to how these mutations could result in cataract. Further work is needed to investigate whether this is the mechanism by which other γD crystallin mutations cause cataract.

1.5.1.2 Lens fibre membrane proteins

Connexin gap junctions are found in the cell membranes of anterior lens epithelial cells and lens fibre cells. Proteins known as major intrinsic protein (MIP) of the lens are the principal protein constituent (50%) of fibre cell membranes, but are absent from epithelial cells.[66] MIP forms water channels or aquaporins. Lens epithelial cell membranes also contain Na^+/K^+ ATPase pumps and transporter proteins for glucose and ascorbate. All these membrane proteins are vital for maintaining lens homeostasis. Mutations in the genes encoding them could theoretically cause cataract. Indeed, mutations in both connexins and in aquaporin 0, the lens aquaporin, have been associated with ADCC.

1.5.1.2.1 Connexins

Connexins are gap junction proteins.[67] Gap junctions are relatively non-specific intercellular membrane channels. Molecular movement through them occurs by passive diffusion. Gap junction channels allow selectivity of passage based principally on molecular size, allowing the movement of molecules smaller than 1000 Daltons, such as cyclic AMP, but preventing the movement of proteins or

nucleic acids. This allows small informational molecules to be directly transmitted between cells. Consequently, this type of communication is an important mechanism for regulating events between cells in embryogenesis.[68]

Apart from a few terminally differentiated cells, such as skeletal muscle, red blood cells, and circulating lymphocytes, most cells in normal tissues generally possess and communicate via gap junctions. These junctions exist in almost all organisms, both vertebrates and invertebrates. In humans, at least twenty connexin genes have been associated with several different diseases including genetic deafness, skin disease, peripheral neuropathies, heart defects and cataracts.[69]

Connexins are a multigene family of proteins. Two alternative nomenclature systems, one based on the molecular mass of the connexin polypeptide and the other based on evolutionary considerations are currently in use.[70] For simplicity sake, the former classification system is used in this thesis. The lens expresses three distinct connexins, connexin 43 (a 43kDalton protein), connexin 46 (a 46kDalton protein) and connexin 50 (a 50kDalton protein), all of which appear to have different functions in maintaining lens homeostasis.[69] Connexin 43 (Cx43) is expressed mainly in lens epithelial cells, while connexin 46 (Cx46) and connexin 50 (Cx50) are expressed in lens fibre cells.[71-73] Mutations in Cx46 and Cx50 have been reported in association with ADCC.

Gap junctions exhibit a hierarchy of assembly.[67] The principal structural component is the membrane protein connexin. Six connexins combine to form a connexon. An individual connexon from one cell docks or associates with a

corresponding connexon on a neighbouring cell to form a gap junction channel, and multiple channels, in turn, cluster or aggregate in the plane of the membrane to form gap junction plaques. The properties of the gap junction channels are defined by the connexins. The different connexin isoforms may associate with each other in many different combinations. This could significantly influence properties such as permeability and gating of the gap junction channels that are formed. Connexons may be homomeric (composed of six identical connexin subunits) or heteromeric (composed of more than one species of connexins). Connexons associate end to end to form a double membrane gap junction channel. The channel may be homotypic (if connexons are identical) or heterotypic (if the two connexons are different). In lens fibres, Cx46 and Cx50 pair up with each other in this way.[71]

Seven and four different cataract-causing mutations in *CX46*[31-33;35;36;38] and *CX50* [34;37;39;74] respectively have been reported to date. All but one of the eleven mutations have been associated with pulverulent congenital cataracts, either predominantly in the nuclear or the lamellar regions of the lens. All connexins have four transmembrane domains and two extracellular loops with cytoplasmic N and C termini. The previously reported mutations associated with congenital cataracts are summarised in table 1.

Table 1 Previously reported mutations in connexin genes associated with cataract			
Gene	Mutation	Location	Reference
CX46	1137insC	C-terminal cytoplasmic tail	Mackay *et al*, 1999
	N63S	First extracellular loop	Mackay *et al*, 1999
	P187L	Second extracellular loop	Rees *et al*, 2000
	F32L	First transmembrane domain	Jiang *et al*, 2003
	P59L	First extracellular loop	Bennett et al, 2004
	R76H	First extracellular loop or second transmembrane domain	Burdon *et al*, 2004
	N188T	Second extracellular loop	Li *et al*, 2004
CX50	P88S	Second transmembrane domain	Shiels *et al*, 1998
	E48K	First extracellular loop	Berry *et al*, 1999
	I247M	C-terminal cytoplasmic tail	Polyakov *et al*, 2001
	R23T	N-terminal cytoplasmic tail	Willoughby *et al*, 2003

Mutations in connexins can affect gap junction function in several ways. The mutation may prevent normal intracellular trafficking of the connexin protein to the cell membrane. Alternatively, the mutant protein may traffic to the cell membrane but its function may be affected. Functional gap junction channels may not be formed. The properties of the gap junction channel may be affected so that the molecular selectivity of the channel or its voltage gating are changed.

Two main approaches have been used to demonstrate localisation of connexin proteins. Firstly, antibodies to the specific connexin have been used to localise it by immunohistochemistry.[75-77] Secondly, fusion proteins composed of connexin and a fluorescent tag have been expressed in connexin-deficient cell lines.[77-79] A major advantage of the latter approach is that it is easy to identify which cells have been transfected and, since they do not need to be fixed, function can be assayed in the live fluorescently tagged cells.

Functional work has been performed on the N63S and 1137insC *CX46* mutants.[80] Voltage-clamp studies were used to assay function of the expressed connexins. Neither of the two mutant connexins was able to form intercellular channels when expressed in paired Xenopus oocytes. The mutants were also impaired in their ability to form hemi gap junctional channels. When each of the mutants was coexpressed with wild-type connexins, both mutations acted like "loss of function" rather than "dominant negative" mutations, because they did not affect the gap junctional conductance induced by either wild type Cx46 or wild type Cx50. Using the same expression system, the functional effect of the P88S mutation in *CX50* was investigated.[81] The mutant connexin again failed to form functional gap junctional channels when paired homotypically. However, this time the mutant functioned in a dominant negative manner when co-expressed with wild-type Cx50. Only one of these mutant subunits is necessary per gap junctional channel to abolish channel function.

The Xenopus oocyte system is rapid and sensitive but requires the blocking of endogenous connexin expression using antisense technology.[82] Mammalian cells selected for transfection studies that do not express functional levels of connexin protein[83;84] offer a more native system to study the behaviour of mammalian connexins. Initial functional work on *CX46* mutants was performed in Xenopus oocytes. Since that time the effects of mutations on connexin function have been investigated by microinjecting tracer dyes into transfected cells and monitoring for dye transfer to adjacent transfected cells through connexin gap junctions.[77-79;83;85-89] A variety of cell types have been transfected with connexins for dye

injection studies including human HeLa cells,[77-79;83;88-90] rat PC12 cells,[88] monkey COS-7 cells,[88] Neuro2A cells,[89] Xenopus oocytes[78] and a keratinocyte cell line.[85] Localisation of the connexin proteins has been established using fluorescent protein tagging of the connexins or immunohistochemistry utilising connexin antibodies.[75-77;83;85-90]

1.5.1.2.2 Aquaporin 0 or major intrinsic protein (MIP)

Aquaporin 0 or MIP is a member of the aquaporins, a ubiquitous family of membrane water transport proteins that allow rapid movements of water across cell membranes.[91] Aquaporin 0 is expressed in lens fibres. Two different mutations in it have been associated with ADCC.[92] The missense mutation T138R has been reported in association with a polymorphic cataract phenotype.[17] The cataract consisted of bilateral discrete progressive punctuate lens opacities limited to mid and peripheral lamellae with additional asymmetric polar opacification. The missense mutation E134G resulted in non-progressive lamellar cataract. *In vitro* functional analyses concluded that neither mutant protein is trafficked to the cell membrane possibly because of protein misfolding during synthesis in the endoplasmic reticulum.[66] Both mutants interfere with the targeting of the wild-type protein thus explaining the dominant-negative effect of the mutations.

1.5.1.3 Transcription factors

Transcription factors are proteins required by RNA polymerases for recognition of specific stimulatory sequences in eukaryotic genes. Transcription factors may activate or repress transcription. They play an important role in the embryological

development of the lens. They control the critical interaction between the embryonic surface ectoderm and the budding optic vesicle, which is essential for normal lens induction.[93] They are expressed in a sequential fashion before and after contact between the optic vesicle and the surface ectoderm. The lens and the anterior segment are linked developmentally with a commonality of origin in surface ectoderm. In addition, there is a necessity for the separation of the corneal and lens surfaces for anterior chamber formation and a requirement for signalling from the lens for anterior segment organization.[94] It is therefore not surprising that mutations in several transcription factor genes including *PITX3*,[95] *PAX6*,[96] *FOXE3*,[97] *EYA1*[98] and *MAF*[99;100] have been implicated in AD congenital cataract and anterior segment mesenchymal dysgenesis (ASMD). Most mutations are associated with ASMD and cataract in all affected individuals within the family although typically there is phenotypic variability. A single family with a mutation in *MAF* has been reported in which some affected members of the family had both ASMD and cataract, while others had isolated cataract.[99;100] Another transcription factor gene, *HSF4*,[26] has been reported to cause isolated cataract in all affected individuals within a large family. Prior to the work described in this thesis, cataract causing mutations in *PITX3* had only been reported in two families.[95] One was a large family with a 17bp duplication mutation causing cortical cataracts and ASMD in all of the affected individuals. The second was a small family with a missense mutation resulting in total cataract. Both affected individuals from the small family developed glaucoma at a young age. The glaucoma may have been inherited as a form of mild ASMD.

1.5.1.4 Cytoskeletal proteins

The structural framework of lens fibres is determined by the interaction of the cytoskeleton and cytoplasmic crystallins. Lens cells possess microfilaments, microtubules, and intermediate filaments. Microfilaments form a cytoskeleton, facilitate changes in cell shape, strengthen cell-to-cell contacts and define plasma membrane compartments. The microtubule cytoskeleton directs intracellular transport processes as well as contributing to the distribution of organelles. The intermediate filaments aid lens cells in overcoming physical stresses including lens accommodation.

1.5.1.4.1 Beaded filament structural protein 2 (BFSP2)

Secondary lens fibre differentiation is accompanied by the replacement of standard cytoskeletal elements by two intermediate filament proteins, BFSP2 and filensin, which assemble into a novel structure known as the beaded filament. A deletion mutation, ΔE233, in the *BFSP2* gene has been reported in association with AD congenital nuclear and sutural cataracts.[101;102] It seems highly likely that mutations in filensin may lead to ADCC.

1.5.2 Loci associated with ADCC

At least six distinct further loci have been associated with ADCC (See Table 1).

At least two different genes cause the three cataract phenotypes on chromosome 1p.[43]

Table 1 Further loci for ADCC

The causative genes within these loci have not yet been identified

Locus	Cataract phenotype	Reference
1pter-p36.1	Nuclear and sutural	Eiberg *et al*, 1995
1p36	PPC	Ionides *et al*, 1997
1pter	Total	McKay *et al*, 2005
2p12	Nuclear	Khaliq *et al*, 2002
15q21-q22	Nuclear and sutural	Vanita *et al*, 2001
17p13	APC	Berry *et al*, 1996
17q24	Cerulean	Armitage *et al*, 1995
20p12-q12	PPC	Yamada *et al*, 2000

1.6 Genotype-Phenotype Correlation

As more genetic mutations causing ADCC have been identified, it has been possible to build up a picture of the relationship between genotype and phenotype. Although it is possible to classify ADCC on the basis of phenotype, it is not possible to correlate phenotype to genotype with certainty. For example, two loci on 1p36[21] and 20p12[23] and mutations in one gene, *CRYAB* on 11q21,[20] have been reported in association with PPC. A particular cataract phenotype, in this case PPC, may not be related to a unique genotype. PPC shows genetic heterogeneity. Similarly, a particular cataract genotype does not always have a unique cataract phenotype - phenotypic heterogeneity. For example, mutations in *CRYGD* have been reported in association with cerulean (P23T),[63] aculeiform (R58H),[60] nuclear (W156X)[62] and lamellar (P23T)[62] cataract. In the case of the P23T mutation,

different phenotypes, cerulean or lamellar may arise from the same mutation. Environmental, or more likely other genetic modifiers, may play a role in determining the eventual phenotype in a particular family.

However, some patterns do emerge. For example, ten out of eleven mutations reported in connexin genes, *CX46* and *CX50*, result in pulverulent cataract.[31-39;74] It is possible that the mutation which has been reported to cause nuclear cataract[74] actually causes nuclear pulverulent cataract. In terms of lens biology, the fact that connexin mutations all result in powdery opacification throughout lens fibres is consistent with the function of connexins, which form gap junctions between adjacent lens fibres. If the function of gap junctions is impaired pre- and post-natally, it follows that lens homeostasis and hence transparency will be affected. Another example is seen with *CRYGD* mutations. Although a number of phenotypes have been reported in association with *CRYGD* mutations, two similar rare cataract phenotypes have only ever been reported in association with mutations in *CRYGD* – aculeiform due to an R58H mutation[60] and prismatic due to an R36S mutation.[64] In both cases, the crystal-like appearance of the cataract reflects the biological malfunction caused by the mutation. These mutations reduce the solubility of γD-crystallins resulting in crystal formation within the lens.[64;65]

1.7 Purpose of research on ADCC

Cataract is the commonest cause of blindness in the world.[1] It is a major public health problem. The only treatment currently available is surgical cataract extraction, most often combined with intraocular lens implantation. The majority

of cataracts are age related. The commonest type leading to surgery is the nuclear type. Research into nuclear cataracts has focused mainly on environmental factors. Age, female sex, and smoking seem to be the most important risk factors. Although environmental risk factors for age related cataract are well-established,[103] age related cataract also has a strong genetic component to its aetiology.

The study of twins has been described as the "perfect natural experiment" in which to determine the relative importance of genetic and environmental factors.[104] To estimate the heritability of a feature, the concordance or correlation of the feature between identical (monozygotic) twins and non-identical (dizygotic) twins is measured, and the magnitude of the concordance or correlation in the two types of twins is compared. The amount of nuclear cataract in a large sample of adult female twins was determined in order to estimate the relative role of genes and environment in the causation of such cataracts.[105] The results showed that 48% of the variation in severity of nuclear cataracts was due to genetic factors. Age accounted for 38% and other environmental factors for 14% of the variance. So, there is an important genetic component to age related cataract. It is likely that several different genes are involved. Sequence variants that are not pathogenic individually may cause an increased likelihood of cataract if present in combination with others. Providing statistical evidence of these associations will form the basis of unravelling the genetic nature of age related cataract.

The identification of the genetic mutations underlying congenital cataract and subsequent functional studies will improve our understanding of normal lens

development and the mechanisms of cataractogenesis. This information, although important, is unlikely to lead to any major clinical advance in the prevention of congenital cataract as the cataracts in this age group are present from birth. However, gaining an understanding of the molecular mechanisms underlying congenital cataract will give us more clues as to the genes that cause age related cataract. The genes responsible for congenital cataract provide candidate genes and a rich source of possible polymorphisms that may contribute to aetiology of age related cataract. Although surgery for adult cataracts is generally associated with good visual outcomes, supply is unable to meet demand on a worldwide scale.[106] With an increasingly ageing population, this discrepancy will only worsen. Therefore, potential non-surgical treatments based on the understandings gained from work such as this, may be able to prevent visual disability from cataracts becoming significant enough to warrant surgery in many people's lifetimes, so alleviating an enormous public health problem.[107]

1.7 Aims of this thesis

In context to the above, the specific objectives of this thesis are to undertake genetic mapping of a cataract locus in a large family from Honduras, to undertake functional characterisation of the gene mutation, to characterise the phenotype and undertake genotyping in two large English families with ADCC and to add further families to the existing inherited cataract panel.

2.0 Methods

2.1 Patients

2.1.1 Ethical approval

Approval for these studies was obtained from The Moorfields Eye Hospital Research Ethics Committee (project numbers BHAS1011 and MOOA183) and from the Institutional Review Board of Self Regional Healthcare IRC, Greenwood, USA.

2.1.2 Consent

All individuals taking part in the studies gave fully informed written consent.

2.1.3 Phenotyping

Both affected and unaffected individuals underwent full clinical examination. This included comprehensive ophthalmic examination, with careful slit lamp examination and anterior segment photography where appropriate. A standard pro-forma was used. Individuals were phenotyped using the classification system which was outlined in the introduction. Pedigrees were constructed using Cyrillic version 2.1.3 software (FamilyGenetix Ltd., Oxford, UK).

2.1.4 Blood sample collection and storage

Peripheral blood samples were collected. In order to obtain blood samples from young children, EMLA cream 5% (AstraZeneca, Macclesfield, UK) was applied to the skin for one hour prior to venepuncture. Blood sample tubes were accurately labelled and stored at 4°C until DNA extraction took place.

2.1.5 DNA extraction and storage

In general, DNA was extracted not more than one week after blood samples were collected. Extraction was carried out by laboratory technicians using the Nucleon BACC2 kit (Tepnel Life Sciences PLC, Manchester, UK). After extraction, DNA was stored at 4°C if for immediate use or at -20°C if for longer term storage.

2.2 Genotyping

2.2.1 Family panel creation

For each large cataract family, a family panel was created. DNA from each available family member was stored in numbered wells of a deep-well plate at 4°C. Once the optimum DNA concentration for polymerase chain reaction (PCR) was established using positive control DNA, the family panels were made up, diluting the DNA to this optimum concentration. The original DNA concentrations were measured using an Eppendorf Biophotometer version 1.26 spectrophotometer (Eppendorf, Hamburg, Germany). The samples were then diluted in the deep wells with deionised water to the optimum DNA concentration, which was found to be 25ng/µl. Finally, the family DNA panel was tested by performing PCR on the panel and examining the size and intensity of each product band after agarose gel electrophoresis.

2.2.2 Polymerase chain reaction (PCR)

PCR for genotyping was carried out using the following standard reaction mixtures:

Table 2 PCR reaction mix for separate forward (FP) and reverse primers (RP)

Mix for 10 reactions	10xNH_4 buffer	dNTP 25mM	$MgCl_2$ 50mM	FP 20µM	RP 20µM	Taq 5U/l	dH_2O	DNA + mix
Volume / µl	25	2	6	3	3	1	200	2 + 20

Table 3 PCR reaction mix for combined primers

Mix for 10 reactions	10xNH_4 buffer	dNTP 25mM	$MgCl_2$ 50mM	Primer 5µM	Taq 5U/l	dH_2O	DNA + mix
Volume / µl	20	1.6	6	10	1	200	2 + 20

The components were mixed together before finally adding 2µl of each panel DNA to 20µl of reaction mixture.

PCR was carried out on Techne Touchgene gradient PCR blocks (Jencons, Leighton Buzzard, UK). After initial denaturation at 95°C for three minutes, samples were processed through 35 cycles of amplification consisting of 30 seconds at 92°C (denaturing), 30 seconds at the optimum annealing temperature for the particular primers, typically 55°C (annealing) and 30 seconds at 72°C (extension). The final extension step at 72°C was lengthened to 5 minutes.

When genotyping was performed using primers from the ABI PRISM linkage mapping set version 2.5 (Applied Biosystems, Warrington, UK), an annealing temperature of 55°C was used in the first instance. When other primers were used, the optimum annealing temperature was first established with control DNA by

performing PCR at a gradient of temperatures and assessing the size and intensity of the products on an agarose gel.

2.2.3 Agarose gel electrophoresis

1% agarose gels were prepared, adding a few drops of 0.5mg/ml ethidium bromide prior to pouring the gels. Gel electrophoresis was performed, after which PCR products were visualised and compared against standard DNA ladders in the Gene Genius gel imaging system (Syngene, Cambridge, UK) using Genesnap 4.0 software (Syngene). A qualitative assessment of the amount of DNA in each PCR product was made by comparing the size and intensities of the bands on the gel. A quantitative estimation of the amount of DNA was made where appropriate by comparing PCR product bands with those of a quantitative DNA ladder.

2.2.4 Genotyping

Fluorescently labelled microsatellite markers were used to perform PCR on family DNA panels. The PCR products were loaded onto an ABI PRISM 3100 genetic analyser (Applied Biosystems) which was configured for genotyping. Automated allele scoring was achieved using Genotyper software version 3.7 (Applied Biosystems). A typical genotyping tracing is shown in Appendix 6.1 The alleles were entered into the pedigrees using Cyrillic software. Two point LOD scores were calculated using the FASTLINK package.[108]

2.2.5 Genotyping strategies

Both whole genome scanning and exclusion of known cataract loci were used to establish linkage. PCR products were pooled to allow simultaneous multiple marker analysis by the genotyper. On average, a family panel was genotyped with three or four markers every day.

2.2.6 Primers used for genotyping

Fluorescently labelled microsatellite primers from the ABI linkage mapping set version 2.5 were used for whole genome scanning and, where possible, for candidate loci exclusion. When a known cataract locus was not excludable using an ABI marker, the Ensembl database (EMBL-EBI and Sanger Institute, both Cambridge, UK) at www.ensembl.org was used to search for alternative adjacent microsatellite markers. The Ensembl database was also used when selecting markers for refining a region of linkage.

2.3 Sequencing

2.3.1 PCR

PCR was performed to amplify the region of DNA to be sequenced. The same PCR block settings as described above were used. A pre-prepared PCR reaction mixture, ReddyMix PCR master mix (Abgene, Epsom, UK), was used.

Table 4 PCR reaction mixture for sequencing

Mix for 1 reaction	ReddyMix	dH_2O	FP 20μM	RP 20μM	DNA
Volume / μl	20	20	1	1	2

PCR was performed at the optimum temperature for the particular pair of primers. The product was checked on a 1% agarose gel. The next stage of sequencing was to clean the PCR product.

2.3.2 PCR product clean up

DNA was extracted from PCR product using the Quickstep 2 PCR purification kit (Edge Biosystems, Gaithersburg, MD, USA). The DNA was then cycle sequenced.

2.3.3 Cycle sequencing

Big Dye Terminator Ready Reaction Mix (Applied Biosystems) was used for cycle sequencing.

Table 5 Reaction mix for cycle sequencing

Mix for 1 reaction	dH_2O	Buffer	Big dye	Primer 20μM	DNA
Volume /μl	4.3	2.5	0.5	0.7	2.0

A standard cycle sequencing program on the PCR blocks was used. After initial denaturation at 95°C for three minutes, samples were processed through 25 cycles consisting of ten seconds at 96°C, five seconds at 50°C and four minutes at 60°C. The sequenced product was then cleaned.

2.3.4 Sequenced product clean up

Several steps were involved in cleaning up the sequenced product. 1µl of sodium acetate was added to precipitate the buffer. 25µl of 100% ethanol was added to the mixture, before freezing it at -20°C for 10 minutes. It was then centrifuged at 13000 rpm for 20 minutes. 300µl of 70% ethanol was added, before centrifuging again at 13000rpm for 20 minutes. The supernatant was carefully removed, leaving a pellet at the bottom of the tube. The tube was oven dried at 60°C for 10 minutes to leave a completely dry pellet. The pellet was thoroughly mixed in 10µl of formamide, before loading the mixture onto an ABI PRISM 3100 Sequencer (Applied Biosystems).

2.3.5 Sequence analysis

DNA sequencing analysis software version 3.7 (Applied Biosystems) was used to view the sequences. The following programmes in the DNAstar Lasergene software package were used to analyse the sequences: EditSeq, Seqman II and MapDraw.

2.3.6 Primers

The entire coding region of the connexin 46 gene (*CX46*) was sequenced using the same pairs of overlapping primers as used by Jiang et al:[32]

Table 6 Primers for sequencing *CX46*

Primer name	Primer sequence
GJA31AF	CTGCGATGCCTGTCCTGTGG
GJA31AR	TTGTCCTGCGGTGGCTCCTT
GJA31BF	CGCCCACCCTCATCTACCT
GJA31BR	GTGGGAACCCGATGGCAAC
GJA31CF	AGCTCAAGCAGGGCGTGACC
GJA31CR	CAAGGGCGGCTGGTGCATCT
GJA31DF	CCCCGGCGCTCAAGGCTTAC
GJA31DR	AACCCTTGTCCCCGCCACCC

2.4 Construct preparation for expression studies

Once a mutation in the connexin 46 gene (*CX46*) was found to segregate with ADCC in a large family, constructs were prepared to investigate the effect of this mutation on connexin 46 protein (Cx46) expression in connexin-deficient human lymphocyte HeLa cells. Wild type and mutant *CX46* inserts and a wild type *CX50* insert were amplified from genomic DNA and then cloned into a mammalian expression vector pTARGET (Promega, Southampton, UK). To prepare fluorescent fusion protein constructs, wild type and mutant *CX46* inserts were first cloned into pGEM-T Easy vector (Promega) and then subcloned into a C-terminal tagged green fluorescent protein (GFP) mammalian expression vector pEGFP-N1 (Invitrogen Clontech, Paisley, UK). Vector diagrams are shown in Appendix 6.2.

2.4.1 Wild type *CX46* construct

2.4.1.1 PCR

After determining the optimum temperature for PCR using control DNA, the entire *CX46* gene was amplified using DNA from an unaffected individual from the family. ATGGGCGACTGGAGCTTTCTGGGAAGAC was used as the forward primer. The reverse primer used was CTAGATGGCCAAGTCCTCCG. The same PCR reaction mixture and block settings were used as described for PCR for sequencing above. However, since the size of the PCR product in this case was 1.3kilobases (1.3kb), the extension phase of each cycle was lengthened to one and a half minutes.

2.4.1.2 Gel purification of PCR product

The PCR product was run on a 1% low melting temperature agarose gel with both a 1kb and a 10kb ladder in order to separate the DNA from the other components. The 1.3kb band was briefly visualised with ultraviolet light before being cut out of the gel. This band of DNA was further purified using the QIAquick gel extraction kit (Qiagen, Crawley, UK).

2.4.1.3 Estimation of purified insert concentration

5µl of purified insert was run on a 1% agarose gel with a 10kb ladder to estimate the DNA concentration of the insert. The insert concentration was estimated as 30ng/µl.

2.4.1.4 Ligation

A 3:1 insert:vector molar ratio was used. The following equation was used to calculate the appropriate amount of insert to include in the ligation reaction:

((ng of vector x kb of insert)/kb size of vector) x insert:vector molar ratio = ng

((60 x 1.3)/5.67) x 3 = 41ng of insert

Since the estimated insert concentration was 30ng/µl, 1.5µl of insert was used. Positive control and background control ligations were also performed so that the following ligation reactions were set up and incubated overnight at 4°C:

Table 7 Ligation reaction mixtures

Volume / µl	Construct reaction	Positive control	Background control
T4 DNA ligase 10x buffer	1	1	1
pTARGET vector (60ng)	1	1	1
Purified PCR product	1.5	-	-
Control insert DNA (8ng)	-	2	-
T4 DNA ligase (3 Weiss units/µl)	1	1	1
Deionised water	5.5	5	7

2.4.1.5 Transformations

The ligation reactions were transformed into separate tubes of high-efficiency JM109 cells (Promega). These cells were then plated onto pre-prepared agar plates, which were incubated overnight at 37°C.

2.4.1.6 Agar plate preparation

One litre of Lucia Broth (LB) agar was prepared by adding deionised water to the following constituents:

10g tryptone (Fisher Scientific UK Limited, Leics, UK)

5g yeast extract (Oxoid Limited, Basingstoke, UK)

10g analytical reagent grade sodium chloride (Fisher Scientific UK Limited)

15g agar number 1 (Oxoid Limited)

The solution was heated in an autoclave at 121°C for approximately 2 hours, until it was homogeneous. It was then air cooled until its temperature was approximately 50°C. 1ml of each of the following filter sterilised solutions were then added to the agar:

ampicillin (50mg/ml) (Sigma, Poole, UK)

X-gal (Sigma) 20mg/ml in dimethyl formamide

IPTG (100mM) (Gold Biotechnology, Inc., St. Louis, MO, USA).

In a laminar box, the LB/ampicillin/IPTG/X-Gal solution was poured into sterile Petri dishes to a thickness of 5-7mm. Once set, plates were either used directly or stored at 4°C for later use.

2.4.1.7 Colony selection

After overnight incubation, the plates were examined. The pTARGET vector contains a modified version of the coding sequence of the α-peptide of β-galactosidase, allowing blue/white recombinant screening. Successful cloning of an insert into the pTARGET vector interrupted the coding sequence of the α-peptide of β-galactosidase, causing colonies to appear white. These colonies were

easy to distinguish from blue colonies of vector alone, in which the coding sequence was uninterrupted. The positive control plates indicated whether or not transformation had worked and showed the efficiency of ligation. The background control plates allowed determination of the number of colonies resulting from vector self-ligation or undigested vector.

2.4.1.8 Preparing plasmids

White colonies from the wild type construct plates were picked and separately incubated overnight at 37°C with agitation in labelled tubes containing LB with 50mg/ml ampicillin. The following day the Genelute plasmid minprep kit (Sigma) was used to purify plasmids from each tube. In the final stage of plasmid preparation, the plasmids were eluted into 50µl of sterile 1xTris-EDTA buffer at pH 7.0. 50µl of the same plasmid was then eluted using sterile deionised water. The plasmid in deionised water was used for sequencing.

2.4.1.9 Sequencing plasmids

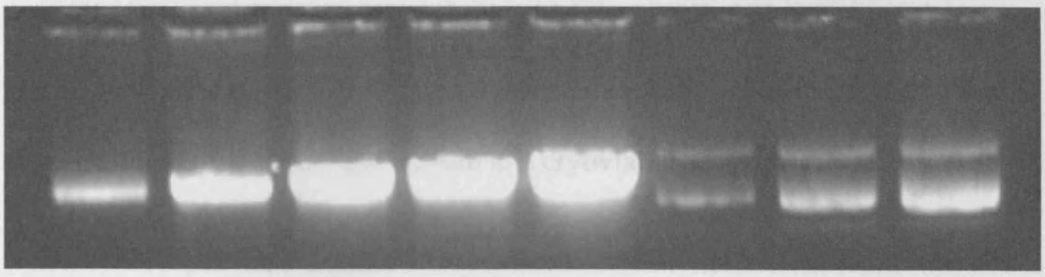
Plasmids were first sequenced with the forward sequencing primer recommended for pTARGET, TAATACGACTCACTATAGGG, to determine which ones had the insert in the correct orientation. Only these plasmids were fully sequenced to check for differences in sequence between the plasmid and the known *CX46* sequence. Sequencing was performed on the purified plasmids as described above from the cycle sequencing stage onwards. Two modifications were made. In the PCR program for cycle sequencing, the initial denaturation at 95°C was lengthened to five minutes. Secondly, 4µl of plasmid DNA was used instead of

2μl of purified PCR product DNA. The volume of water was reduced so as to keep the total reaction mixture volume the same. For sequencing the entire *CX46* gene, further primers were used including the reverse sequencing primer recommended for pTARGET, TTACGCCAAGTTATTTAGGTGACA, a forward internal sequencing primer, GTCCCTATTCCTCAACGTG, and a reverse internal sequencing primer, CACGTTGAGGAATAGGGAC.

2.4.1.10 Estimation of construct concentration

Once a plasmid was identified which consisted of wild type *CX46* correctly cloned into pTARGET, its concentration was estimated. A 1% agarose gel was prepared for gel electrophoresis to run various dilutions of the construct plasmid against known concentrations of Lambda DNA (Promega), a control plasmid (See Figure 2). The band of the 1:20 dilution of construct plasmid was roughly equal in size and intensity to that obtained with 100ng of Lambda DNA. Since 10μl of construct plasmid was loaded, the construct plasmid concentration was estimated as 200ng/μl.

Figure 2 Picture of 1% agarose gel used for estimating insert concentration



100 200 300 400 500 1:40 1:20 1:10

ng of Lambda DNA dilutions of construct plasmid

2.4.1.11 Glycerol stocks

A small volume of the overnight culture which had been used to prepare the plasmid was used to prepare a glycerol stock. 300µl of sterile 50% glycerol was mixed with 700µl of overnight culture in a cryovial. The cryovial was stored at -80°C.

2.4.2 Mutant Cx46 construct

The same cloning process was used to prepare a construct consisting of mutant *CX46* cloned into pTARGET. This time, however, the DNA used to prepare the insert was from an affected individual and the forward primer used for the initial PCR was ATGGGCTACTGGAGCTTTCTGGGAAGAC. When the insert sequence was checked, only those plasmids with the mutation present were selected for full *CX46* gene sequencing.

2.4.3 Wild type Cx50 construct

The forward primer, ATGGGCGACTGGAGTTTCCTGGGGAAC, and the reverse primer, TCATACGGTTAGATCGTCTGAC, were used to prepare the insert from an unaffected individual's DNA. The cloning process was otherwise the same as that for wild type Cx46 construct.

2.4.4 Fluorescent fusion protein constructs

To prepare fluorescent fusion protein constructs, PCR products of the entire *CX46* gene were first cloned into pGEM-T Easy vector (Promega) and then subcloned

into pEGFP-N1 vector (BD Biosciences Clontech, Oxford). The primers used for creating the wild type insert for pGEM-T were forward primer AAGCTTATGGGCGACTGGAGCTTTC and reverse primer GGATCCTTGATGGCCAAGTCCTCCG. The same reverse primer was used for creating the mutant insert. The forward primer used for this was AAGCTTATGGGCTACTGGAGCTTTC. Subcloning was achieved by performing double digests on the PGEM-T constructs and on the pEGFP-N1 vector using Hind III and Bam HI restriction enzymes (both Promega). The double digests on the PGEM-T constructs were performed by incubating the following reaction mixtures at 37°C for 4 hours.

Table 8 Double digest reaction mixtures for PGEM-T Easy vector construct

	dH_2O	10x buffer	BSA 1µg/µl	Construct DNA 400ng/µl	Hind III	Bam HI
Volume / µl	11	2	2	3	1	1

The double digest on the pEGFP-N1 vector was performed by incubating the reaction mixture at 37°C for 15 minutes and then at 65°C for 15 minutes to inactivate the shrimp alkaline phosphatase (SAP) (Promega). SAP dephosphorylated the vector in order to prevent self-ligation.

Table 9 Double digest reaction mixture for pEGFP-N1 vector

	dH_2O	10x buffer	BSA 1µg/µl	Vector 1µg/µl	Hind III	Bam HI	SAP 1u/µl
Volume / µl	10	3	3	1	1.5	1.5	10

After the double digests, the mutant and wild type inserts were gel purified. Their concentrations and that of the digested dephosphorylated vector were estimated on a 1% agarose gel before the inserts were each ligated into the vector using a 3:1 insert:vector ratio. Positive control and background control ligations were performed as before. Transformants were plated onto agar plates containing kanamycin (50mg/ml) (Sigma, St. Louis, MO, USA) instead of ampicillin because pEGFP-N1 vector is resistant to kanamycin unlike pTARGET vector which is ampicillin-resistant. Since pEGFP-N1 vector does not allow blue/white colony selection, colonies were screened for successful cloning of the insert into pEGFP-N1 by carrying out colony PCR using GJA31CF and GJA31CR primers at their optimum temperature of 55°C. Plasmid minipreps were prepared using colonies which contained the inserts. These plasmids were sequenced to confirm that the fusion protein sequences were correct. A wild type fusion protein construct (CX46-GFP) and a mutant fusion protein construct (D3Y-GFP) were prepared and glycerol stocks of them were prepared and stored as described above.

2.5 Protein expression studies: localisation

Human HeLa cells were cultured for forty-eight hours in culture medium comprising 88.5% DMEM (Sigma), 10% foetal calf serum (Labtech, Ringmer, UK), 0.5% penicillin 100U/ml (Sigma), 0.5% streptomycin 100µg/ml (Sigma) and 0.5% fungizone 1.25µg/ml (Sigma). The cultures were incubated at 37°C in 5% CO_2. The cells were then seeded onto coverslips at a density of approximately 50% for twenty-four hours prior to transfection. Connexin 46 wild type (cx46wt), connexin 46 mutant (cx46mt), CX46-GFP and D3Y-GFP constructs and positive control CX26-GFP construct[77] (a gift from D.L. Becker) were transfected into

adherent human HeLa cells using Effectene transfection reagent (Qiagen). Cells were cultured for a further twenty-four hours. Those cells transfected with GFP construct were visualised directly at this stage using a Leica DMLB fluorescence microscope with water-dipping lenses (Leica Microsystems, Milton Keynes, UK). Images were digitally recorded with a Leica DCF300 camera. Those cells transfected with cx46wt or cx46mt constructs were fixed with 4% paraformaldehyde and immunostained. Fixed HeLa cells were briefly rinsed with phosphate-buffered saline (PBS, pH 7.4) and then immersed in blocking solution (100mM L-lysine in 1xPBS with 0.05% Triton X-100) at room temperature for one hour. Cells were then incubated with primary rabbit antibodies against Cx46[72] (a gift from T.W. White) diluted 1:1000 in blocking solution at 37°C for one hour. Following three five minute washes with PBS, cells were incubated with 1:500 CY3, rhodamine-conjugated goat anti-rabbit IgG (Zymed, San Fransisco, CA, USA), in blocking solution at 37°C for one hour. After three further five minute washes with PBS, preparations were mounted in Citifluor (Canterbury,UK) on slides and sealed with nail varnish. Slides were examined on a Leica fixed stage epifluorescence microscope and on a Leica SP2 laser scanning confocal microscope and images were stored digitally.

2.6 Protein expression studies: function

Dye injection studies were carried out using glass micropipettes pulled from thick walled borosilicate capillary glass (Harvard Apparatus, Holliston, MA, USA) with a Sutter Instruments horizontal pipette puller (Novato, CA, USA). Coverslips of HeLa cells expressing GFP were transferred into larger dishes containing Neurobasal medium (Gibco, Rockville, MD, USA). The cells were visualised on a

Leica DMLFS fixed stage epifluorescence microscope using water-dipping lenses so that pairs or groups of GFP positive cells could be selected for microinjection. Single cells from each group were iontophoretically injected with a mixture of 5% lucifer yellow (Sigma) and 2% neurobiotin (Vector, Peterborough, UK). Cells were observed for two minutes for intercellular passage of lucifer yellow. Relevant images were recorded digitally with a Leica DCF300 camera. To reveal the distribution of neurobiotin, preparations were fixed with 4% paraformaldehyde, permeabilised and blocked with 100mM L-lysine in 1xPBS with 0.05% Triton X-100 for one hour and then stained with 1:200 Avidin-CY3 (Zymed) in blocking solution for thirty minutes. Fluorescent signals were examined on a Leica DMLB fluorescence microscope and images were stored digitally using a Leica DCF300 camera.

3.0 Results

3.1 Phenotyping and linkage studies in two large families

3.1.1 Phenotyping

Two large English Caucasian families with ADCC were identified from the genetic clinic database at Moorfields Eye Hospital. The pedigrees are shown in Figures 3 and 4. AD inheritance is supported in both families by the presence of affected male and female individuals in each generation and male-to-male transmission. Previously, the families were predominantly phenotyped for cataract and other clinical abnormalities. The affected proband from family A (subject III:5), a 41 year-old female, was re-phenotyped as part of this study. She had a unique phenotype for her family comprising PPC and ASMD. Her most recent operation under general anaesthetic was digitally recorded to aid phenotyping since it allowed more accurate assessment than was possible when she was awake due to her nystagmus. Her unaffected son (IV:5) was re-phenotyped in the light of her unusual phenotype. Her affected eight year-old daughter (IV:6) and affected fifteen year-old niece (IV:7), who had both not been phenotyped previously, were also examined. Blood samples were collected for DNA extraction from all of these individuals. Phenotyping and blood sample collection was performed on three individuals from family B. An affected 35 year-old mother (IV:11) and her two children, an affected seven year-old daughter (V:3) and an unaffected eleven year-old son (V:4), were examined. By seeing the affected daughter (V:3), the family's phenotype was defined since the phenotype was progressive and so in order to define it accurately, it was important to see it in its early stages.

Figure 3 Pedigree A.

Square symbols denote males; circles denote females; affected individuals are denoted by black symbols.

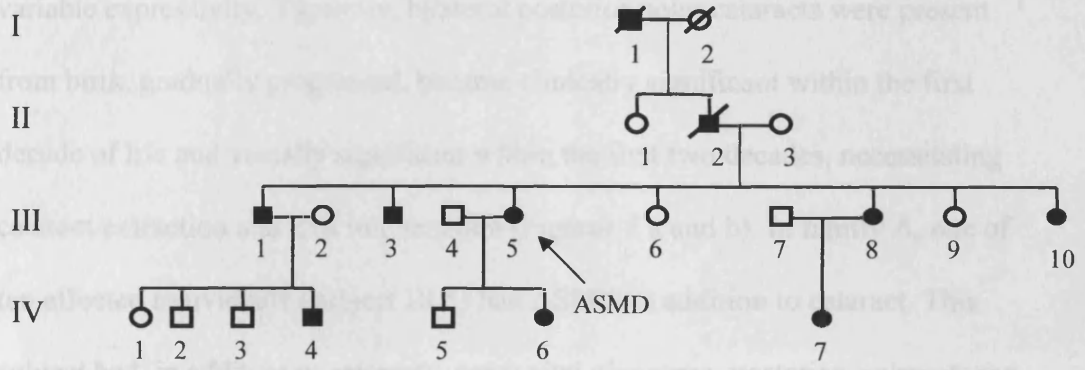
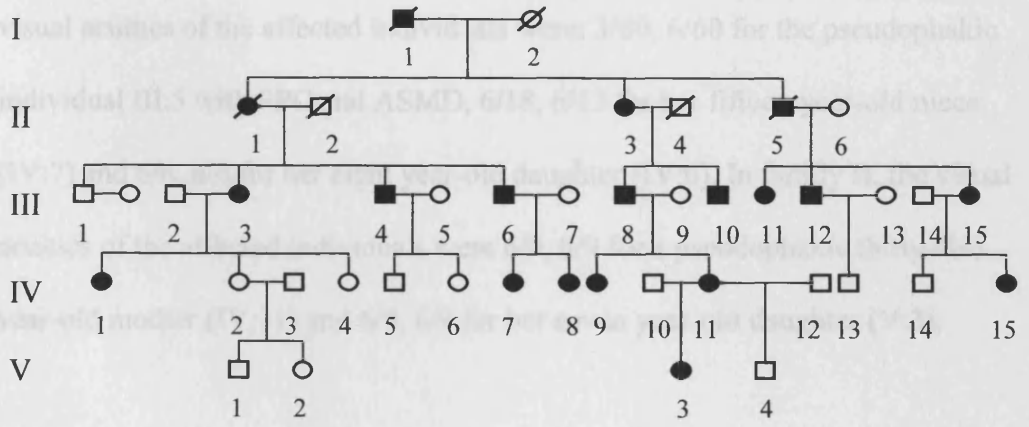


Figure 4 Pedigree B

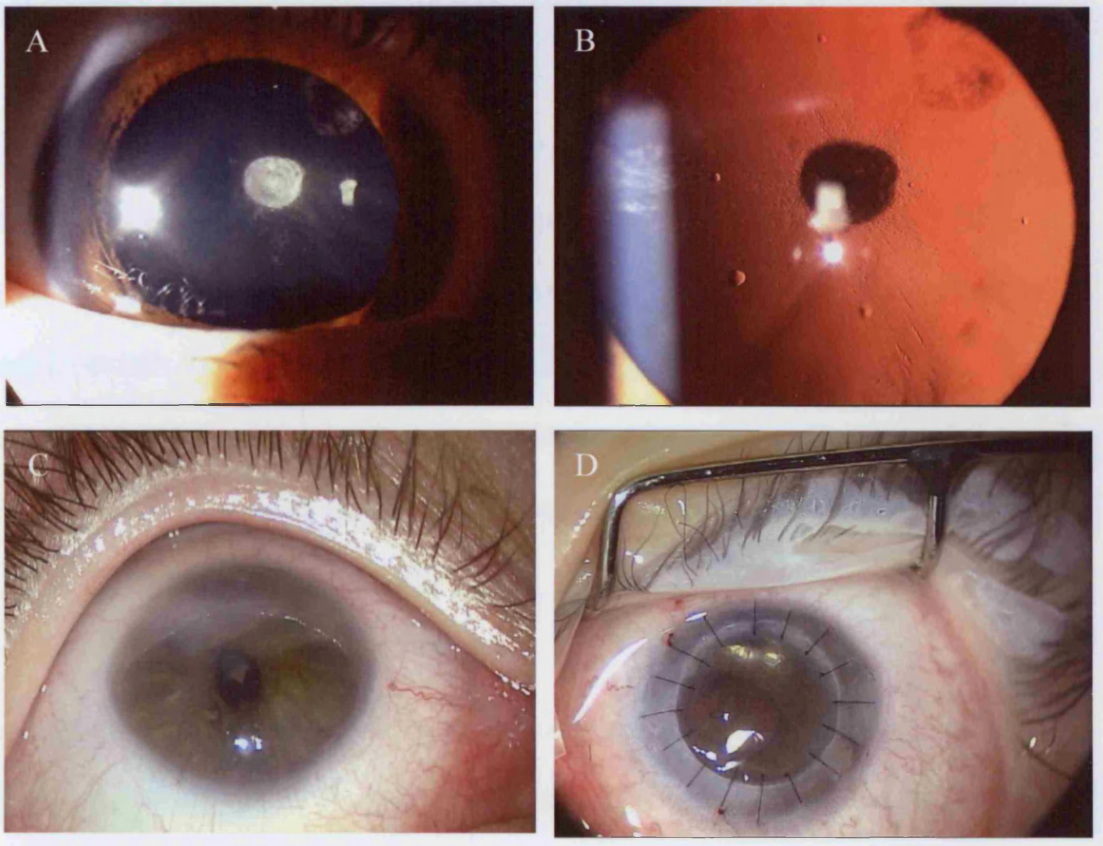


3.1.2 The phenotype

Overall, the age ranges of the individuals examined were: eight to seventy (family A) and seven to eighty (family B). All affected individuals from the two families had the same cataract phenotype. PPC was fully penetrant but exhibited some variable expressivity. Typically, bilateral posterior polar cataracts were present from birth, gradually progressed, became clinically significant within the first decade of life and visually significant within the first two decades, necessitating cataract extraction and lens implantation (Figures 5 a and b). In family A, one of ten affected individuals (subject III:5) had ASMD in addition to cataract. This subject had, in addition to cataracts, congenital glaucoma, posterior embryotoxon, atrophic irides and microcornea. She underwent a left penetrating keratoplasty aged 28 and repeat penetrating keratoplasty aged 41 for endothelial decompensation in the previous graft (Figures 5 c and d). There were no other ocular or systemic abnormalities in individuals from either family. Visual acuities were recorded for all the individuals examined in this thesis. In family A, the visual acuities of the affected individuals were: 3/60, 6/60 for the pseudophakic individual III:5 with PPC and ASMD, 6/18, 6/12 for her fifteen year-old niece (IV:7) and 6/6, 6/6 for her eight year-old daughter (IV:6). In family B, the visual acuities of the affected individuals were 6/9, 6/9 for a pseudophakic thirty-five year-old mother (IV:11) and 6/9, 6/9 for her seven year-old daughter (V:3).

Figure 5 Family A phenotypes

(a) Direct illumination and (b) retroillumination slit lamp photographs of posterior polar cataract in individual IV:7 from family A. (c) Right and (d) left eyes of subject III:5 from family A at the end of repeat left penetrating keratoplasty.



3.1.3 Linkage studies

Initial linkage analysis was previously performed for family A. After excluding the following candidate loci for PPC: 1p36, 11q21 and 20p12, whole genome scan had revealed a significantly positive two-point LOD score (3.61 at recombination fraction, θ, of 0) for the marker D10S192. In this thesis, the region of linkage was refined. Using markers from the ABI linkage mapping set version 2.5 and other specifically ordered markers in the region, maximum two-point LOD scores of 3.61 at θ=0 were obtained at seven other markers in the region including D10S564 and D10S597. The linked region was refined by demonstrating flanking crossovers with an upper crossover at D10S1686 and a lower crossover at D10S1693 (See Figure 6). This region encompassed the *PITX3* gene. Appendix 6.3 shows a LOD score table for the region.

Whole genome scan of family B had previously indicated possible linkage to the long arm of chromosome 10. In this thesis, the tentative linkage was supported using multiple markers around the *PITX3* gene, including those shown in Figure 7. A maximum two-point LOD score of 2.58 at θ=0 was obtained for marker D10S1739. Haplotyping suggested that families A and B are unrelated. For example, for marker D10S192, the disease haplotype in family A is allele 4, whilst in family B it is allele 5.

Direct sequencing, undertaken by Dr Berry,[22] demonstrated that all affected individuals in both families had an identical mutation in the *PITX3* gene (shown in Appendix 6.4) and that this mutation was absent in unaffected individuals and 100 normal controls. Sequence analysis of the gene revealed a 17 bp duplication

Figure 6 Family A with haplotype data shown

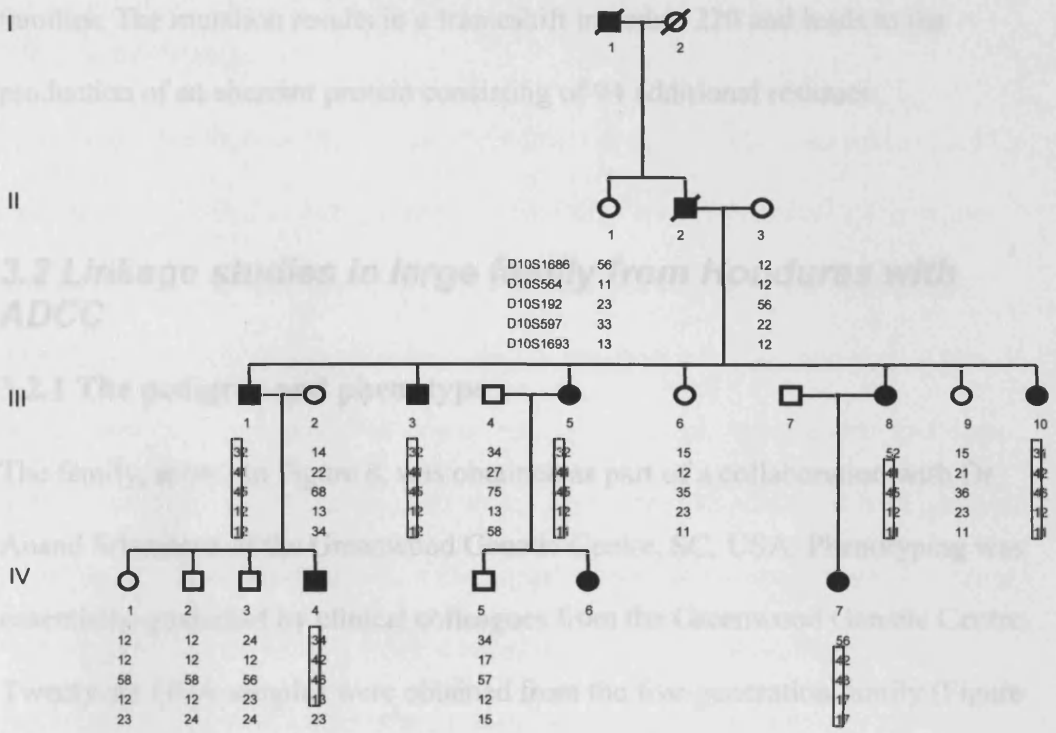
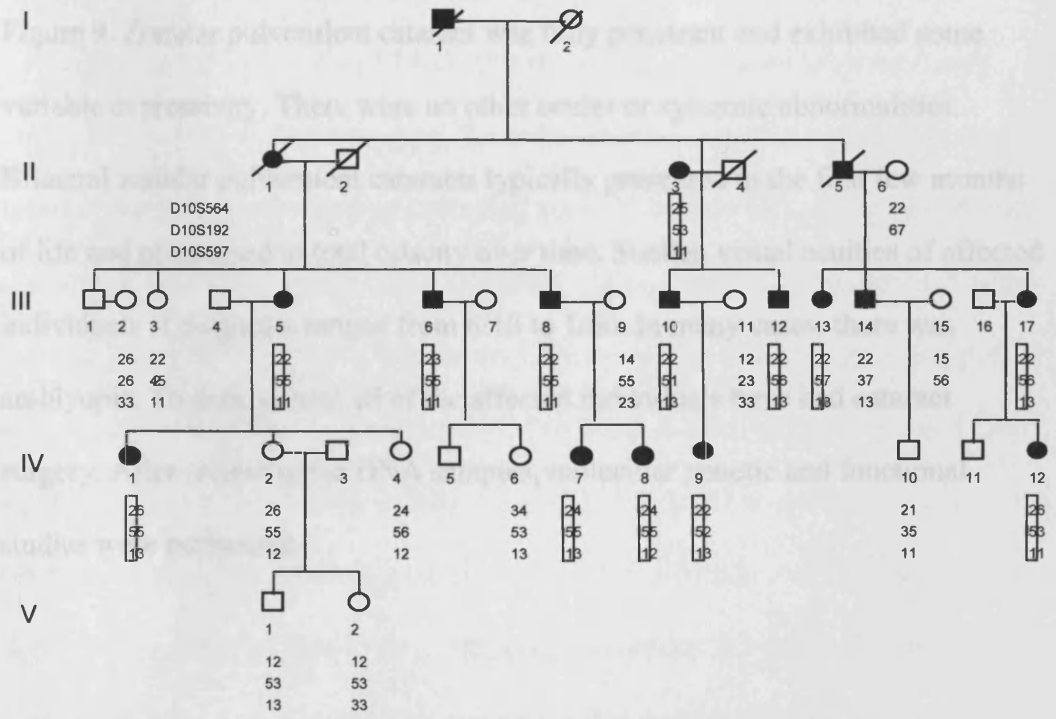


Figure 7 Family B with haplotype data shown



mutation in exon 4 which segregated with the disease phenotype in the two families. The mutation results in a frameshift in codon 220 and leads to the production of an aberrant protein consisting of 94 additional residues.

3.2 Linkage studies in large family from Honduras with ADCC

3.2.1 The pedigree and phenotype

The family, shown in Figure 8, was obtained as part of a collaboration with Dr Anand Srivastava of the Greenwood Genetic Centre, SC, USA. Phenotyping was essentially conducted by clinical colleagues from the Greenwood Genetic Centre. Twenty-six DNA samples were obtained from the five-generation family (Figure 8) from Honduras with ADCC. AD inheritance is supported by the presence of affected individuals in each of the five generations, equal numbers of affected males and females and male-to-male transmission. The phenotype is shown in Figure 9. Zonular pulverulent cataract was fully penetrant and exhibited some variable expressivity. There were no other ocular or systemic abnormalities. Bilateral zonular pulverulent cataracts typically presented in the first few months of life and progressed to total opacity over time. Snellen visual acuities of affected individuals at diagnosis ranged from 6/18 to 1/60. In many cases, there was amblyopia. To date, almost all of the affected individuals have had cataract surgery. After receiving the DNA samples, molecular genetic and functional studies were performed.

3.2.2 Genotyping

Twenty-six members of the family, including sixteen affected individuals and ten unaffected individuals, were genotyped. Initially, the only clinical information provided by our collaborators was that affected individuals in the family had ADCC. The phenotype was not described. Therefore, whole genome scan was begun since linkage to a known or a novel cataract locus was possible and there were no phenotypic clues to suggest which known loci to screen. Whole genome scan began with chromosome 1. After a total of twenty-eight markers were used, the whole of chromosome 1 was excluded in terms of linkage. Since whole genome scan was time-consuming, it was decided to change strategy and exclude known cataract genes on other chromosomes by genotyping using markers close to these genes. Several candidate genes implicated in ADCC were excluded by linkage analysis (LOD scores less than 1 at θ=0) including *CX50*, *CRYGC*, *CRYGD*, *BFSP2, MIP* and *PITX3.* At this stage, our clinical collaborators sent more information on the phenotype. Since the phenotype was zonular pulverulent cataract, the priority changed to excluding genes known to be associated with this phenotype. *CX50* had already been excluded. *CX46*, known to be associated with zonular pulverulent cataracts, was investigated next. The family was genotyped using marker D13S175, adjacent to the *CX46* gene. A maximum two-point LOD score of 2.53 was obtained at θ=0 for D13S175. The LOD score was not above 3 since this marker was relatively uninformative in the family. All the other markers in this region from the ABI linkage mapping set version 2.5 were used in an attempt to find a more informative marker for this family. The family was genotyped using D13S1236, D13S1316, D13S1275, D13S1243, D13S1304,

D13S217 and D13S292. These markers were also relatively uninformative in the family. Maximum two-point LOD scores of 1.03 at $\theta=0$ and 1.58 at $\theta=0.05$ were obtained for markers D13S1316 and D13S292 respectively. The LOD score table is shown in Appendix 6.5. Haplotyping refined the region of possible linkage (Figure 8) as being between the tip of chromosome 13q above and the marker D13S1275 (the lower crossover) below. It was decided that the LOD score of 2.53 for marker D13S175 together with correlation between the family's phenotype and the phenotype typical of cataracts associated with *CX46* mutations warranted screening of this gene.

Figure 8 Abbreviated pedigree for family C

Square symbols denote males; circles denote females; affected individuals are denoted by black symbols. Haplotype data is shown.

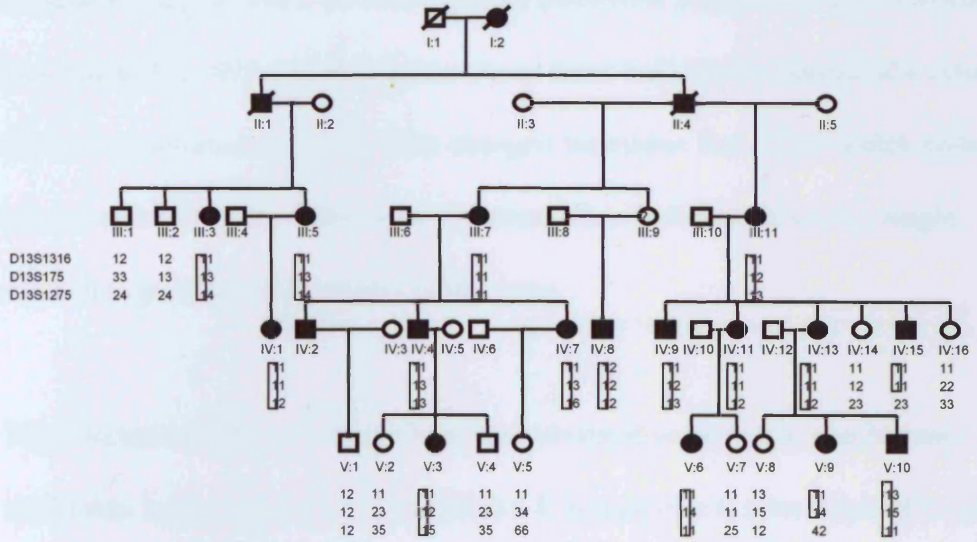
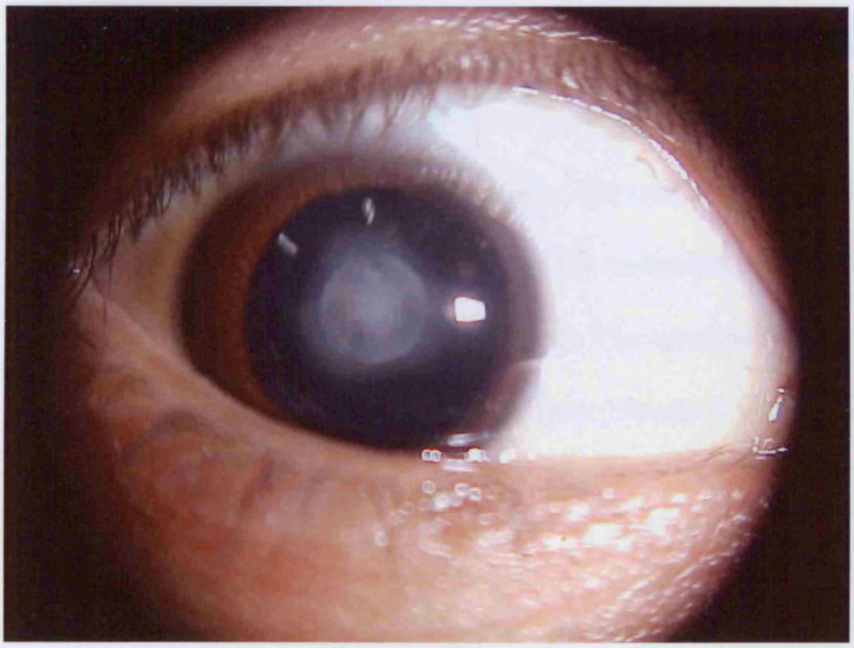


Figure 9 Typical zonular pulverulent cataract from family C



3.3 Identification of a novel mutation in CX46

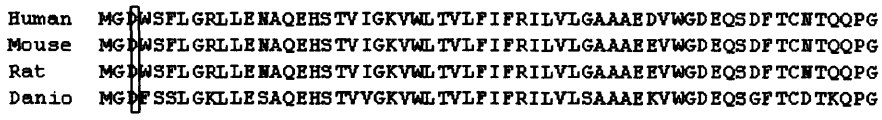
The entire *CX46* gene (GenBank reference NM_021954) was directly sequenced in an affected and an unaffected individual from family C. Both the affected and the unaffected individual differed from the published sequence in the following way. Nucleotide 895 was an adenine (A) in these individuals instead of a cytosine (C) in the published sequence. This changed the codon from CTG which codes for leucine to ATG which codes for methionine. This is, in fact, a known single nucleotide polymorphism (SNP) in this gene.

The affected individual differed from the published sequence in another way. At nucleotide 7, the affected individual had a T instead of a G (See Appendix 6.6). This mutation changes the amino acid encoded by the third codon from a negatively-charged amino acid aspartate (D) to an uncharged amino acid tyrosine (Y). This variant 7G>T, causing a novel heterozygous missense mutation D3Y, was identified by direct sequencing in all 16 affected individuals but in none of 10 unaffected individuals from the family nor 106 control chromosomes from a control panel in our laboratory. Additionally, our collaborators in America tested for the mutation and found it was absent in 124 control chromosomes (32 Hispanic, 70 white, 16 black and 6 unknown). The tyrosine residue is conserved across species represented in GenBank (Figure 10) and in other related connexins (Appendix 6.7).

The D3Y mutation is likely to be causative since it segregates with affected status throughout the pedigree and is absent both in unaffected individuals within the pedigree and in unaffected, unrelated controls. The mutation results in a

Figure 10 Cross species alignment of Connexin 46

Alignment of residues 1-60 of human Cx46 with mouse, rat and zebra fish (*Danio rerio*) orthologues is shown. D3 is boxed.



significant amino acid substitution, changing a negatively-charged amino acid to an uncharged amino acid close to the amino terminus of the protein.

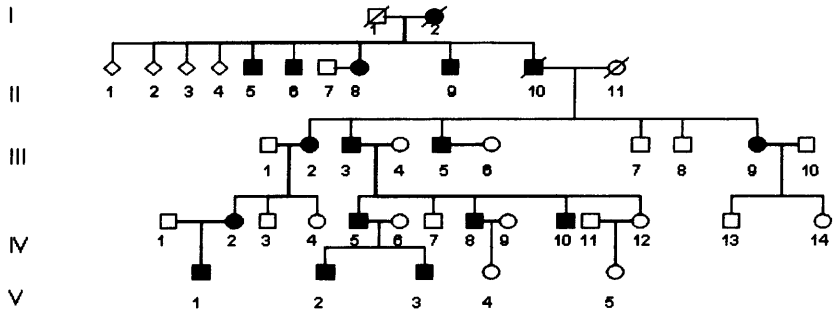
3.4 Phenotyping and screening for the D3Y mutation in another family with ADCC

Three affected individuals (III:2, IV:2 and V:1) from a five-generation English Caucasian family with AD zonular pulverulent cataract, family D, were phenotyped and blood samples were collected for DNA. The pedigree was ascertained through the cataract clinic at Great Ormond Street Hospital (Figure 11). All three individuals had bilateral cataracts from birth. However, there was some intrafamilial variation in cataract severity. Subject IV:2 had cataract surgery on both eyes within her first year of life. Her son, aged three, subject V:1, had mild cataracts and had not yet undergone surgery.

The entire *CX46* gene was sequenced using DNA from individual IV:2. This individual also had the SNP at position 895 (resulting in a leucine to methionine change), as previously seen in the two individuals from the Honduran family. However, there were no other sequence variations anywhere in the gene. DNA from an affected member of this family has therefore been added to the cataract panel for candidate gene screening.

Figure 11 Family D pedigree

Square symbols denote males; circles denote females; affected individuals are denoted by black symbols.



3.5 Protein expression studies on D3Y mutation - localisation

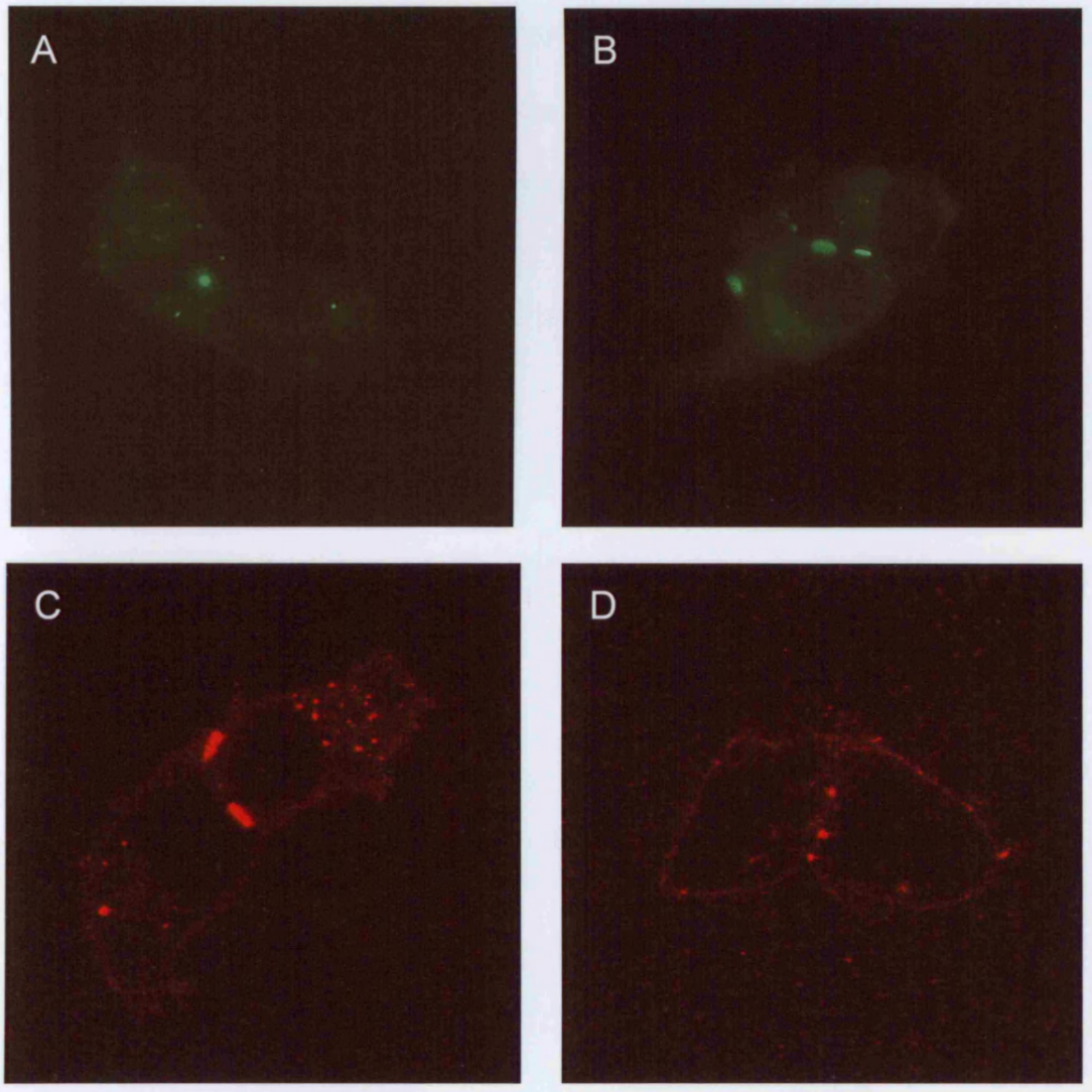
The following construct plasmids were successfully prepared for expression studies:

1. wild type *CX46* in pTARGET vector (cx46wt)
2. mutant *CX46* in pTARGET vector (cx46mt)
3. wild type *CX46* in pEGFP-N1 vector (CX46-GFP)
4. mutant *CX46* in pEGFP-N1 vector (D3Y-GFP)
5. wild type *CX50* in pTARGET vector (cx50wt)

Human HeLa cells were transiently transfected in separate experiments with the first four constructs above containing *CX46*. GFP expression was observed for cells transfected with either CX46-GFP or D3Y-GFP at points of contact between adjacent cells. This indicated appropriate trafficking of the protein and the formation of gap junction plaques between neighbouring transfected cells (figures 12 a and b). For cx46wt and cx46mt constructs, immunolabelling with an antibody specific to connexin 46 protein (Cx46) revealed Cx46 at points of contact between adjacent cells. GFP tagging had clearly not interfered with the protein trafficking (figures 12 c and d). Wild type and mutant connexins both trafficked to the membrane in a similar way. It was anticipated that, time permitting, Cx46 and Cx50 constructs would be co-expressed to mimic the situation in vivo. However, due to time constraints, these experiments were not possible.

Figure 12 Cx46 localisation

CX46-GFP (A), D3Y-GFP (B), Cx46wt (C) and Cx46mt (D) proteins all traffic to the membrane to form plaques between cells. The GFP labelled protein appears green whilst the latter two proteins are immunostained red using a rhodamine-conjugated secondary antibody.



3.6 Protein expression studies on D3Y mutation – function

Dye injection studies were performed on cells transfected with CX46-GFP, D3Y-GFP and CX26-GFP (a gift from D.L. Becker) plasmids in separate experiments. All ten CX26-GFP transfected cells injected with a mixture of lucifer yellow and neurobiotin exhibited lucifer yellow dye transfer to adjacent transfected cells within two minutes. None of ten CX46-GFP transfected cells or ten D3Y-GFP transfected cells exhibited such intercellular transfer with either lucifer yellow or the smaller molecule neurobiotin. These results are summarised in Figures 13 and 14.

Figure 13 Intercellular transfer studies I

Lucifer yellow dye injected into a CX26-GFP transfected cell with an intercellular plaque (A) transfers to the transfected cell above (B) within 2 minutes of injection. No such intercellular dye transfer is seen for cx46wt transfected cells (C, D) or for cx46mt transfected cells (E, F)

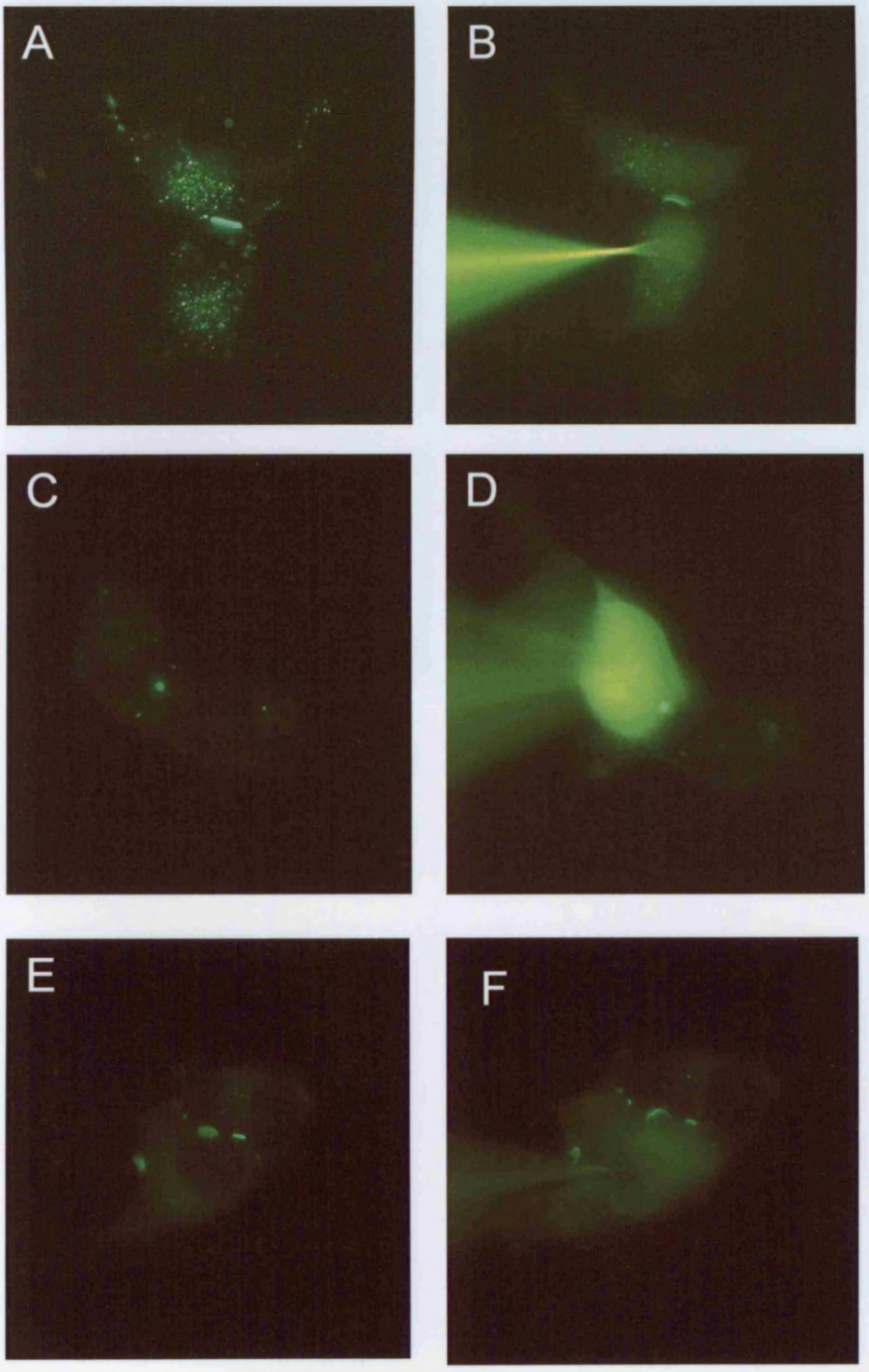
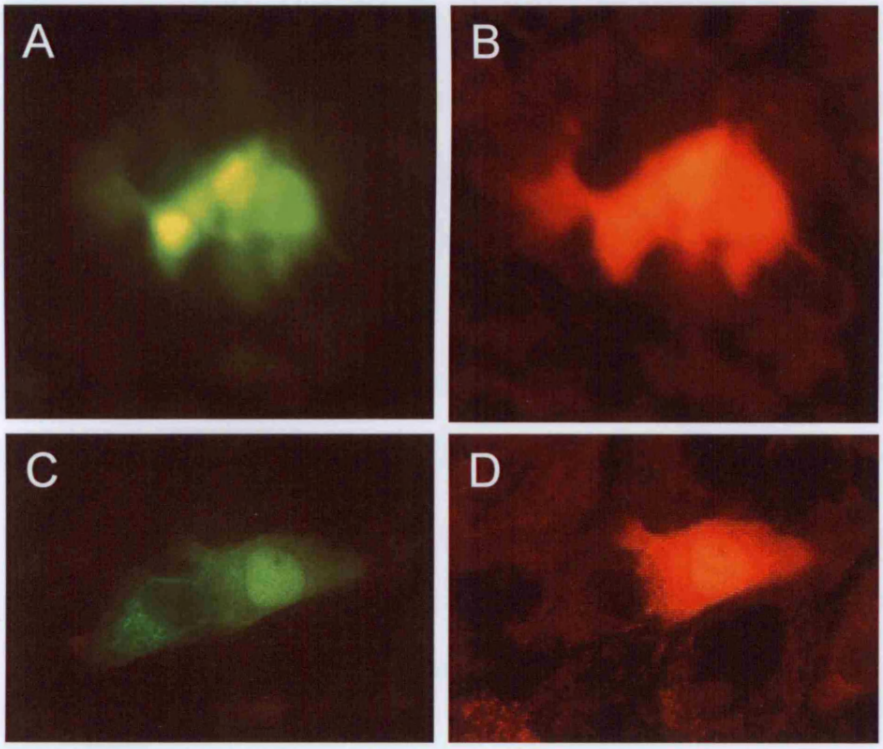


Figure 14 Intercellular transfer studies II

Following dye injection, fixing and staining with Avidin-CY3 to localise neurobiotin, neither lucifer yellow nor neurobiotin was shown to have been transferred between adjacent coupled cx46wt (A, B) or cx46mt (C, D) transfected cells.



3.7 Cataract panel

DNA from twenty-one affected individuals from different families with inherited cataract was added to our existing cataract panel. The families were ascertained through several sources, predominantly the cataract clinic at Great Ormond Street Hospital and the genetic clinic at Moorfields Eye Hospital. All of the individuals added to the panel were phenotyped as well as many of the other individuals in these small families – affecteds, unaffecteds and married-in individuals. Blood samples for DNA were obtained from those individuals who gave their consent. The pattern of cataract inheritance in 90% (19/21) of these mainly small families was AD. Two families, both of which had consanguineous unaffected parents, demonstrated AR inheritance. In many cases, it was not possible to record the cataract phenotype because all the available affected individuals had already undergone cataract surgery and the cataract phenotype was not accurately documented in the hospital notes. However, thirteen of these twenty-one families had a determinable cataract phenotype. Four of these families had PPC, one of which also had ASMD. This family was investigated but not found to have a mutation in the *PITX3* gene, an obvious candidate gene for PPC and ASMD.

Table 10 Summary of the panel families

Inheritance pattern	Cataract phenotype	Number of families
AD	APC	1
AD	PPC	3
AD	PPC + ASMD	1
AD	Nuclear pulverulent	3
AD	Zonular pulverulent	2
AD	Nuclear	2
AD	Unknown phenotype	7
AR	PPC	1
AR	total	1

The pedigrees of these twenty-one families are illustrated in the figures on the following pages, together with relevant photographs from some of the families.

Figure 15 Panel family 1

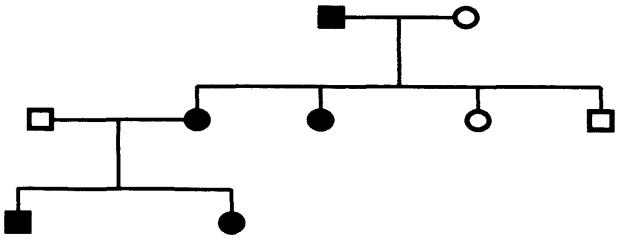


Figure 16 Panel family 2

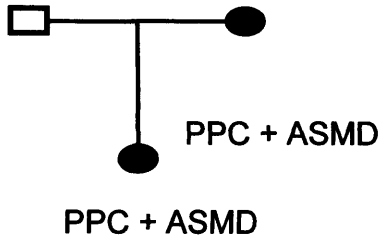


Figure 17 Panel family 3

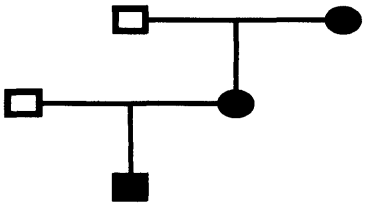


Figure 18 Panel family 4

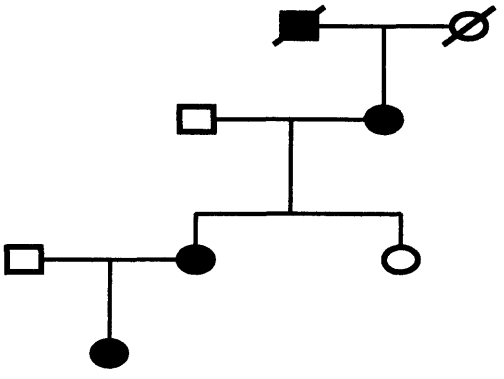


Figure 19 Panel family 5

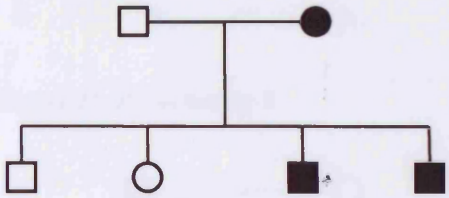


Figure 20 Panel family 6

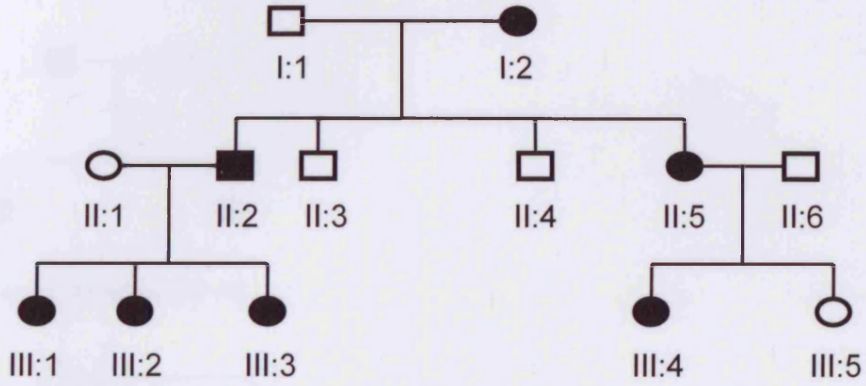


Figure 21 Panel family 6 phenotype

Right eye of subject II:2 from panel family 6, showing the nuclear pulverulent cataract phenotype typical of affected individuals in the family

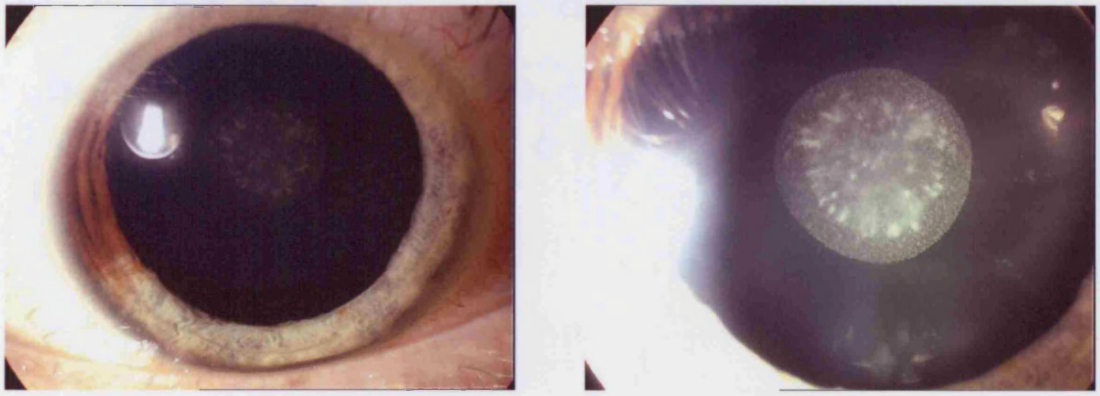


Figure 22 Panel family 7

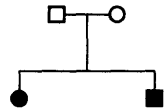


Figure 23 Panel family 8

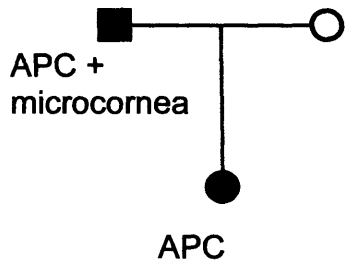


Figure 24 Panel family 9

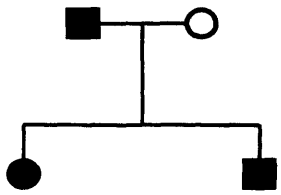


Figure 25 Panel family 10

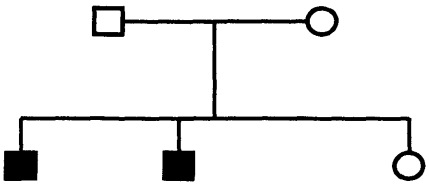


Figure 26 Panel family 11

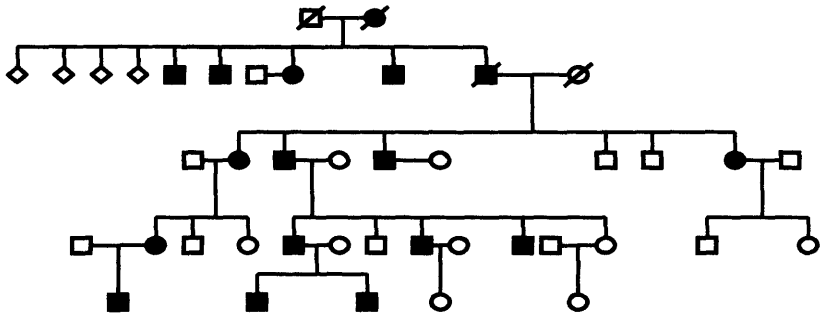


Figure 27 Panel family 12

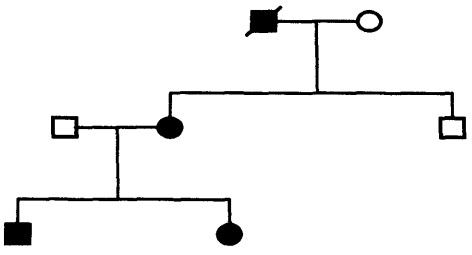


Figure 28 Panel family 13

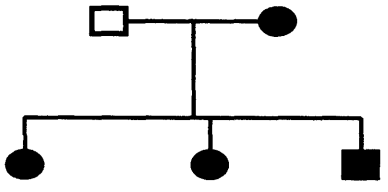


Figure 29 Panel family 14

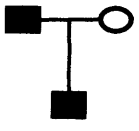


Figure 30 Panel family 15

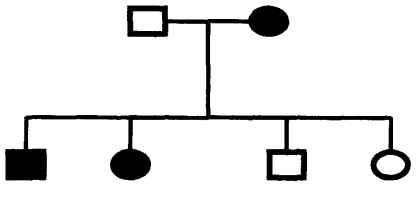


Figure 31 Panel family 16

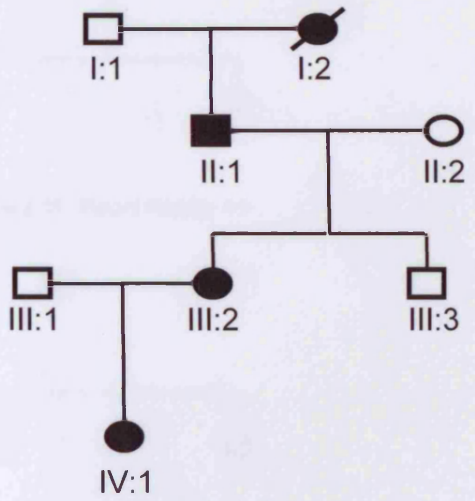


Figure 32 Panel family 16 phenotype

Diffuse illumination (A) and slit beam view (B) of nuclear cataract in subject III:2 from panel family 16, typical of the family's phenotype

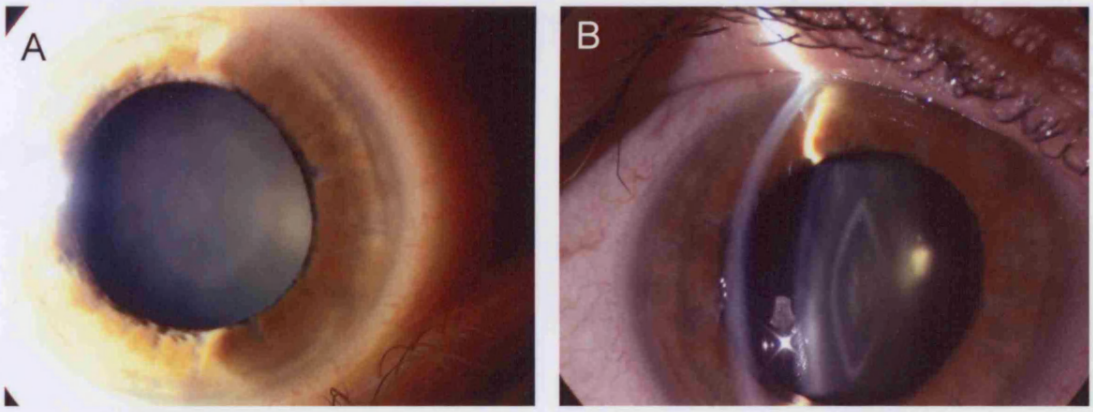


Figure 33 Panel family 17

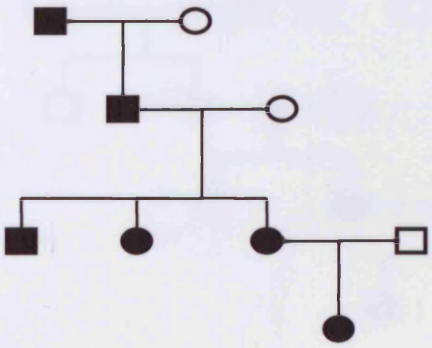


Figure 34 Panel family 18

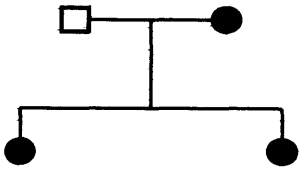


Figure 35 Panel family 19

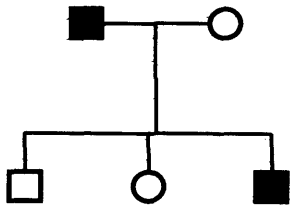


Figure 36 Panel family 20

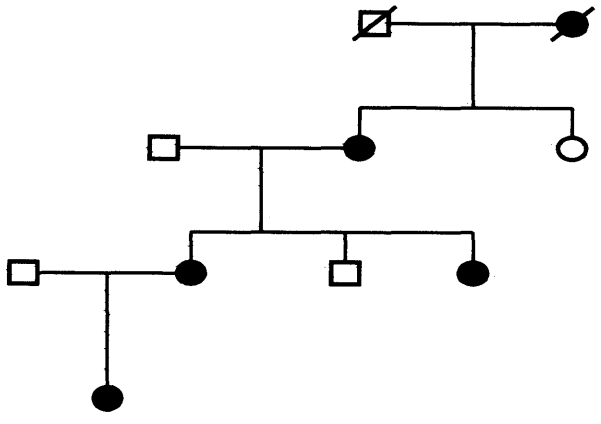
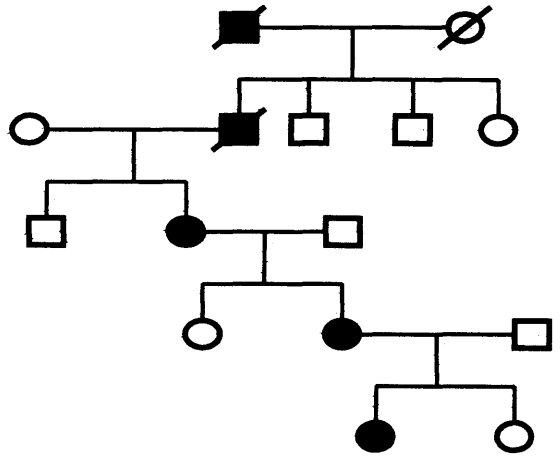


Figure 37 Panel family 21



4.0 Discussion

4.1 Overview of this thesis

This thesis illustrates the wide variety of cataract phenotypes and genotypes in ADCC. ADCC is a term which describes diseases which differ markedly in terms of visual outcome, the necessity and timing of surgical intervention and the underlying molecular mechanisms causing cataract. Understanding the molecular biology of ADCC gives us insights into the aetiology of commoner age related cataract. This thesis adds knowledge to the field of ADCC, both in terms of novel phenotypic consequences of a previously known mutation in *PITX3* and in terms of discovery and functional investigation of a novel mutation in a known cataract gene, *CX46*.

4.2 Novel phenotypic consequences of a mutation in PITX3

PITX3 encodes the transcription factor protein, PITX3. Transcription factors may activate or repress transcription. They play important roles in the embryological development of the lens and the anterior segment of the eye. Mutations in several transcription factor genes including *PITX3*,[95] *PAX6*,[96] *FOXE3*,[97] *EYA1*[98] and *MAF*[99;100] have been implicated in ADCC and ASMD. Most of these mutations are associated with ASMD and cataract in all affected individuals within the family although typically there is phenotypic variability. A single family with a mutation in *MAF* has been reported in which some affected members of the family had both ASMD and cataract, while others had isolated cataract.[99;100] Another transcription factor gene, *HSF4*,[26] has been reported to cause isolated cataract in all affected individuals within a large family.

PITX3 is a member of the *PITX* gene family and encodes a paired-like class of homeobox transcription factors. Both *PITX2* and *PITX3* genes are involved in eye development and are expressed in most developing ocular tissues, including the cornea, lens and retina.[109] Mutations in *PITX2* have been reported in Rieger's syndrome.[110] *PITX3* comprises four exons and encodes a protein of 302 amino acid residues. Sequence analysis of this gene revealed, in exon 4, a 17bp duplication (657-673dup17)[22] that segregated with disease in all the affected members of both families A and B. This 17bp duplication mutation results in a frameshift in codon 220 and leads to the production of an aberrant protein consisting of 94 additional residues. The mutation does not affect the homeodomain and the mechanism by which the aberrant protein leads to cataract and ASMD is unclear. In particular, the mechanism by which mutations in *PITX3* give rise to PPC, such a localised form of lens opacity remains unknown. A better understanding of the disease mechanism will be gained by future functional studies.

In the aphakia mouse mutant, two deletions in the promoter of the homeobox transcription factor *Pitx3* lead to loss of its function and to arrest of eye development at the lens stalk stage.[111] Prior to this thesis, cataract causing mutations in the homologous human *PITX3* gene had only been reported in two families.[95] In both families, mutations caused cataracts in association with ASMD in all of the affected individuals. In one family, the cataract phenotype was cortical cataract. In the other family, the cataract phenotype was total cataract. In large families A and B in this thesis, the same 17bp mutation in *PITX3* as previously reported results in a different phenotype, PPC, in all affected

individuals. Only one out of a total of twenty-four affected individuals in families A and B exhibited features of ASMD. The phenomenon of the same mutation causing a different phenotype in different families has been observed previously for the nonsense mutation, Q155X, in the *CRYBB2* gene. This mutation has been associated with cerulean cataract,[57] cerulean and sutural cataract[45] and nuclear pulverulent cataract[58] in three unrelated families. In families A and B in this thesis, all the affected individuals had PPC. However, one affected individual in family A also exhibited features of ASMD. This variability between individuals carrying the same causative mutation may result from a number of factors including the effects of other modifier genes, intrauterine environmental factors or it may be due to stochastic developmental events.[112]

PPC has not previously been reported in association with mutations in *PITX3*. AD PPC is genetically heterogeneous. Two loci on 1p36[21] and 20p12[113] and mutations in one gene, *CRYAB* on 11q21,[20] have been reported in association with this phenotype. Now, it has been shown that a mutation in *PITX3*, on 10q25, can also give rise to isolated PPC.[22] This adds to the evidence behind our current understanding of the relationship between genotype and phenotype in ADCC. A particular cataract phenotype does not have a unique genotype, as is well illustrated for PPC. The multiplicity of genes causing the same or similar phenotype contributes to the genetic heterogeneity of ADCC. Families A and B illustrate a further feature of the genotype-phenotype relationship. This second phenomenon has been described as clinical heterogeneity indicating that mutations in the same gene may give rise to completely different cataract phenotypes. For example, mutations in *CRYGD* have been reported in association

with cerulean (P23T),[63] aculeiform (R58H),[60] nuclear (W156X)[62] and lamellar (P23T)[62] cataract. In the case of the P23T mutation, even with an identical mutation, the cataract phenotype varies between families. Environmental, or more likely other genetic modifiers, may play a role in determining the eventual phenotype in a particular family. Such modifier gene influences have been identified in a recessive murine cataract and it is likely that similar gene-gene interactions will be identified in human cataract.[114] Clinical heterogeneity is also observed for *PITX3*. The same 17bp duplication mutation that was previously reported to cause cortical cataracts and ASMD in all of the affected individuals,[95] causes PPC with or without ASMD in all affected individuals in families A and B.

Two further families, one of English Caucasian and the other of Chinese origin, which display the same phenotypic consequences of this mutation in *PITX3* have been reported by our group. In total, five genetically distinct large families with mutations in *PITX3* in association with AD PPC were reported by our group.[22] One family, of Hispanic origin, had a novel deletion mutation. The other four families had the same 17bp duplication mutation. In family B and the Chinese family, the phenotype is isolated PPC. In family A and the other English Caucasian family, one of ten affected individuals and four of eleven affected individuals respectively also had ASMD.[24] In this latter family, the anterior segment developmental abnormalities varied from peripheral sclerocornea to complex disorders with changes similar to Peter's anomaly (central corneal opacity with iridocorneal adhesions). So, even amongst individuals within a family who had ASMD, the exact nature of the ASMD varied considerably.

4.3 Novel mutation in connexin 46 gene causing ADCC

Different strategies can be used for mapping a disease depending on the clinical information available. If detailed phenotypic information is unavailable, whole genome scan or exclusion of known loci can be used to map ADCC. These approaches are indicated when there are no phenotypic clues as to which known gene or locus is involved. If the phenotype of ADCC is known, a different approach may be used. Known genes or loci associated with the specific phenotype of cataract are excluded first. Once these have been excluded, other known cataract genes and loci are excluded. If cataract does not map to a known locus, whole genome scan is required.

Whole genome scan and exclusion of known cataract loci approaches were initially used for mapping ADCC in the Honduran family. Once the phenotype in the Honduran family was known, the genes known to cause AD zonular pulverulent were excluded. This lead to markers around the *CX46* gene being genotyped. A relatively high two-point LOD score of 2.53 was found using D13S175, a marker close to *CX46*. This and other adjacent markers were relatively uninformative in the family. However, zonular pulverulent cataract segregated perfectly for this marker and so the *CX46* gene was sequenced. Sequencing revealed a novel mutation which segregated with cataract throughout the family. The effect of the mutation on trafficking and function of expressed connexin 46 protein (Cx46) was investigated.

Connexins are ubiquitous gap junction proteins which allow small informational molecules to be directly transmitted between cells.[67] The lens expresses three distinct connexins.[69] Cx43 is expressed mainly in lens epithelial cells, while Cx46

and Cx50 are expressed in lens fibre cells.[71-73] All but one of the eleven mutations in *CX46* and *CX50* genes that have been reported in ADCC are associated with AD pulverulent cataract, the phenotype in family C. This is the first time a *CX46* mutation has been reported in a family of Hispanic Central American origin. It widens the ethnic diversity of families with *CX46* mutations causing ADCC. Previous mutations have been reported in five families of Caucasian and two families of Chinese ancestry. Screening *CX46* in another large family with AD zonular pulverulent cataract, family D, did not demonstrate a mutation, highlighting the genetic heterogeneity that exists for zonular pulverulent cataract even when connexin mutations almost always cause pulverulent cataracts.

The *CX46* gene encodes a 435 amino acid protein which has four transmembrane domains and two extracellular loops with cytoplasmic N and C termini. The mutation in the Honduran family changes the seventh nucleotide base in *CX46* from a G to a T. This results in the amino acid encoded by the third codon of *CX46* being changed from a negatively-charged amino acid aspartate (D) to an uncharged amino acid tyrosine (Y). This D3Y mutation is the first mutation within the N-terminal cytoplasmic tail region of *CX46* to be associated with congenital cataract. Substitutions in the amino acid residues of the N terminus may interfere with the conformation and flexibility of the amino terminus and also with voltage gating.[115;116] The D3 residue of *CX46* is phylogenetically conserved from zebrafish to man, indicating that the aspartate is likely to be functionally important and that the mutation may therefore have a detrimental physiological effect.

The effect of the novel mutation, D3Y, on Cx46 trafficking was investigated. Both by immunohistochemistry and by fluorescent fusion protein localisation, it was shown that the mutant Cx46 traffics to the membrane identically to wild type Cx46 in human HeLa cells. This strongly suggests that abnormal trafficking of mutant protein is not the mechanism by which it malfunctions in human lens fibre cells, so causing cataract. The mutation is therefore likely to affect intercellular communication through the Cx46 channel.

Dye transfer experiments were performed to test this hypothesis. Lucifer yellow dye transfer was observed between adjacent human HeLa cells transfected with a CX26-GFP positive control plasmid construct but was not observed in cells transfected with either CX46-GFP or D3Y-GFP. This was an unexpected result. It was thought that lucifer yellow or the smaller molecule neurobiotin would transfer between cells transfected with the wild type CX46-GFP construct but not with the D3Y-GFP construct. This would have confirmed the hypothesis that the D3Y mutation affects the function but not the trafficking of Cx46 in human HeLa cells. There are no published reports of lucifer yellow or neurobiotin transfer between adjacent human HeLa cells transfected with CX46-GFP. However, lucifer yellow dye transfer and neurobiotin transfer have previously been demonstrated using human HeLa cells transfected with CX26-GFP and CX30-GFP respectively.[77] In this thesis, lucifer yellow dye transfer was demonstrated for CX26-GFP but neither lucifer yellow nor neurobiotin transferred between cells transfected with CX46-GFP or D3Y-GFP in separate experiments. There are a number of possible explanations for this result.

Firstly, it is possible that neither lucifer yellow nor neurobiotin but that an alternative dye passes through open Cx46 gap junctions. Dye transfer has been demonstrated in Cx46 expressing cells using Alexa350, a coumarin derivative dye (Dr Reiner Eckert, personal communication). Certainly, published data for other connexins suggests that it is not possible to predict which molecules will pass through gap junctions composed of a particular connexin.[77;78;83;117] A second possible explanation for the unexpected results is that the Cx46-GFP protein does not allow the normal functioning of Cx46 gap junctions to take place. The obvious difference between the Cx46 protein expressed in these cells and endogenous Cx46 is the presence of the GFP component of the protein, which allowed transfected cells to be identified. However, GFP tagging has been successfully used previously for other connexins, like Cx26 and Cx30, without affecting function. It is still possible though that Cx46 specifically may be functionally affected by GFP tagging.

Due to time constraints, it was not possible to repeat the dye transfer experiments using an alternative dye such as Alexa350 which could have demonstrated normal wild type function but abnormal mutant Cx46 protein function. Other further and future work is summarised in Appendix 6.8.

4.4 Final Conclusions

The complex role of transcription factors in anterior segment development has been highlighted by finding novel phenotypic consequences of a known mutation in *PITX3*. It is highly likely that the effects of the *PITX3* mutation depend on the genetic background of the family and, in particular, on the effects of other

modifier genes in affected individuals within the family. This adds to the evidence for clinical heterogeneity in ADCC. The fact that PITX3 is a further locus for PPC adds to the evidence for genetic heterogeneity in ADCC.

Finding a novel mutation in *CX46* in a completely different ethnic population to those previously reported but with an almost identical phenotype to all the other known mutations suggests that, in some instances, genotype can play the major role in determining phenotype. Through the investigation of the functional effects of cataract-causing mutations, as we have begun for the D3Y mutation in CX46, further insights will be gained into the fundamental biological events which underlie congenital cataracts. The information we gain from such functional studies should lead to a better understanding of the causes and potential therapies for age related cataract, which remains the commonest cause of blindness in the world.

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Ref Type: Report

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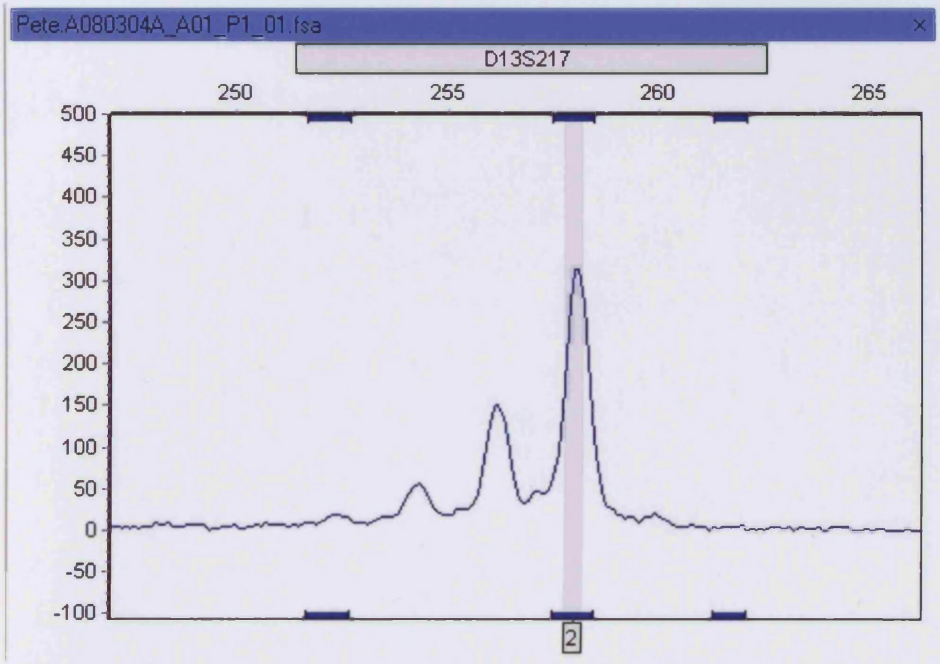
6.0 Appendices

6.1 Genotyping

Figure 38 An example of a genotyper tracing

An individual with 2/2 genotype for marker D13S217 is shown



6.2 Vector diagrams

Figure 39 pTARGET vector circle map and sequence reference points

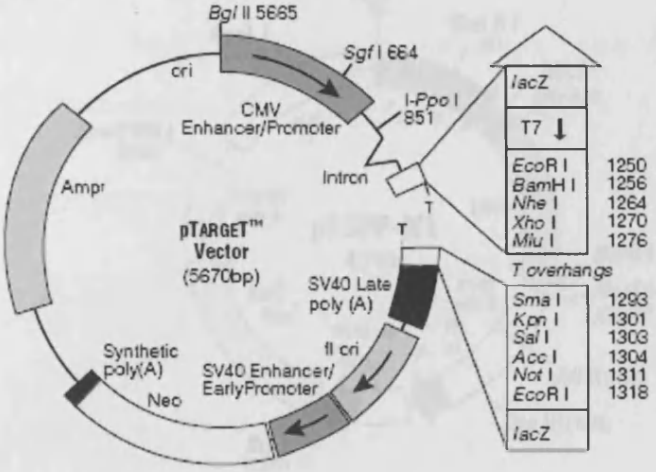


Figure 40 pGEM-T Easy vector circle map and sequence reference points

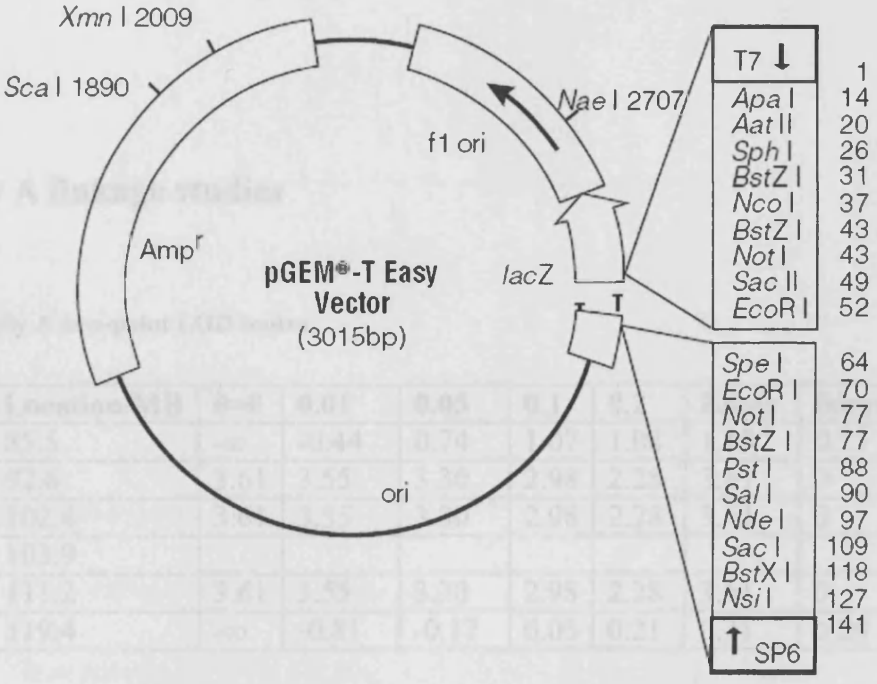
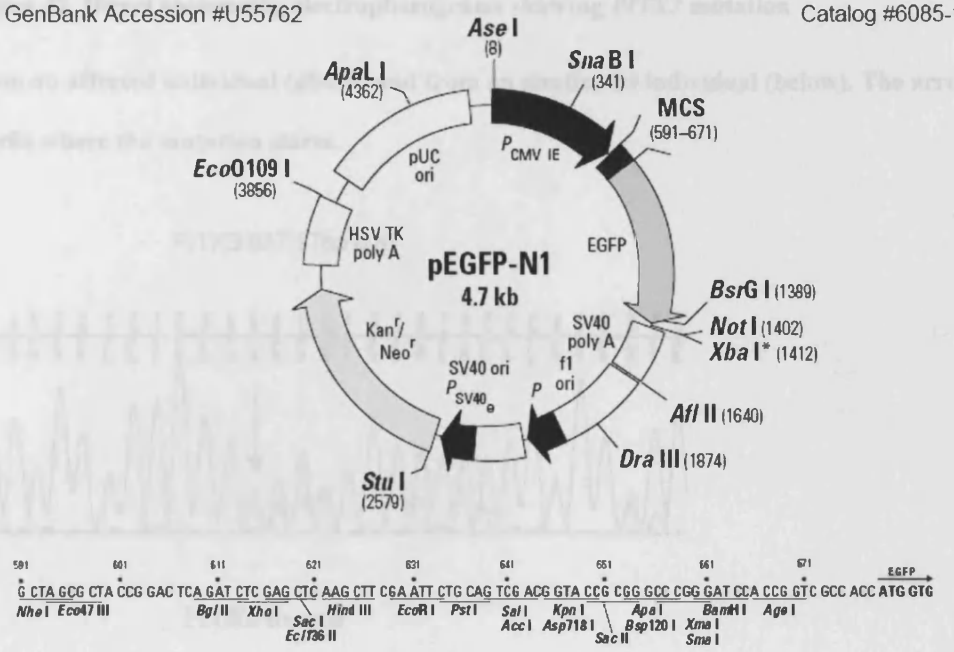


Figure 41 pEGFP-N1 vector information



6.3 Family A linkage studies

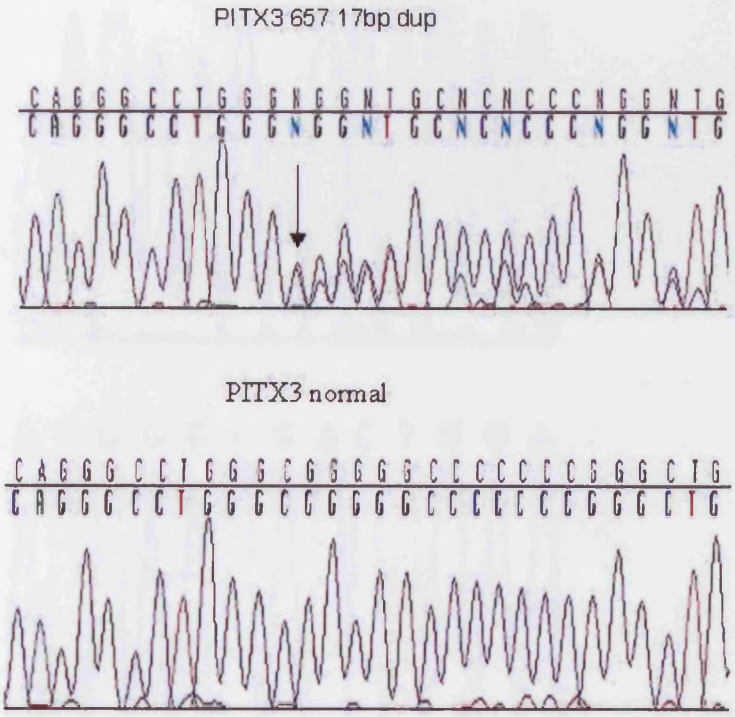
Table 11 Family A two-point LOD scores

Marker	Location/MB	θ=0	0.01	0.05	0.1	0.2	Zmax	θmax
D10S1686	85.5	-∞	-0.44	0.74	1.07	1.08	1.13	0.15
D10S564	92.6	3.61	3.55	3.30	2.98	2.28	3.61	0
D10S192	102.4	3.61	3.55	3.30	2.98	2.28	3.61	0
PITX3	103.9							
D10S597	111.2	3.61	3.55	3.30	2.98	2.28	3.61	0
D10S1693	119.4	-∞	-0.81	-0.17	0.05	0.21	0.21	0.20

6.4 Family A *PITX3* mutation

Figure 42 Direct sequencing electropherograms showing *PITX3* mutation

From an affected individual (above) and from an unaffected individual (below). The arrow marks where the mutation starts.



6.5 Family C linkage studies

Table 12 Family C two-point LOD scores

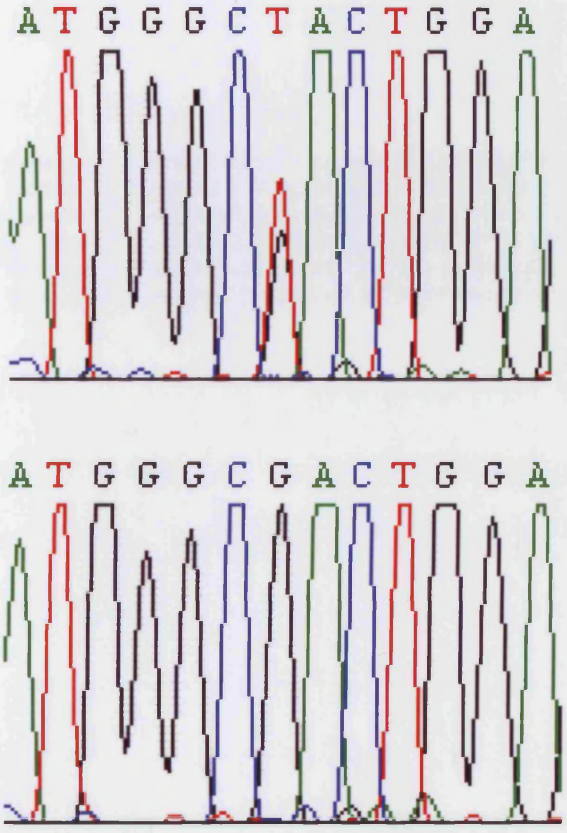
The distance shown is the genetic distance from p-tel

Marker	Distance/cM	θ=0	0.10	0.20	0.30	0.40	Zmax	θmax
D1S1316	0	1.03	0.75	0.48	0.23	0.06	1.03	0
CX46								
D13S175	6.03	2.53	2.31	1.84	1.25	0.62	2.53	0
D13S1275	6.99	-∞	2.73	2.54	1.86	0.94	2.76	0.12
D13S292	7.09	1.42	1.51	1.17	0.73	0.28	1.58	0.05

6.6 Family C *CX46* mutation

Figure 43 Direct sequencing electropherograms showing *CX46* mutation

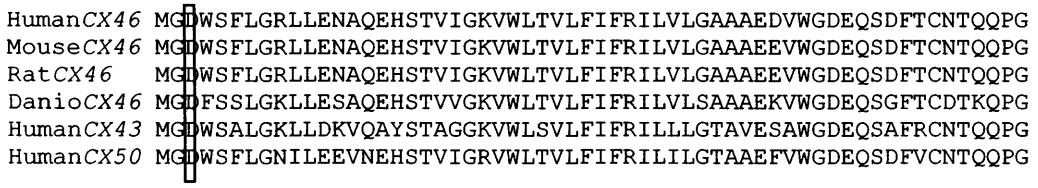
Sequences from an affected individual (above) and an unaffected individual (below)



6.7 Highly conserved third residue in connexins

Figure 44 Cross species alignment of connexin 46 and other connexins

Alignment of residues 1-60 of human Cx46 with mouse, rat and zebra fish (*Danio rerio*) orthologues is shown, together with human Cx43 and Cx50. D3 is boxed.



6.8 Further work

As explained in section 4.3, further work might have demonstrated normal wild type but abnormal mutant Cx46 protein function. In addition, time constraints prevented co-expression of Cx46 and Cx50 constructs which would have simulated in vivo protein expression in the lens. Given that mutations in *CX46* are not uncommon and that twenty-one new families were added to the cataract panel in this thesis, it would have been interesting to sequence affected members of each of these families for mutations in *CX46* as was done for family D.

Inherited childhood cataracts are not uncommon. However, cataract-causing mutations have only been found in relatively few families. Often, the pedigrees illustrate autosomal dominant inheritance and yet mutations in known cataract genes are not found. Given our understanding of lens biology, there do not appear to be many more new candidate cataract genes to screen. However, it is possible that mutations occur in intronic regions of known cataract genes which are currently not sequenced. In the future, intronic cataract-causing mutations may be found.