GENETIC EPIDEMIOLOGY AND MOLECULAR STUDIES OF CONGENITAL BLINDNESS AT FAMILY LEVEL

BY

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DEPARTMENT OF BIOLOGICAL SCIENCES
QUAID-I-AZAM UNIVERSITY
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CERTIFICATE

This thesis, submitted by Miss Farhat Jabeen is accepted in its present form by the Department of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan as satisfying the thesis requirements for the degree of Doctor of Philosophy in Biological Sciences (Genetics).

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Dedicated To
My Adorable Mother
and
Amiable brothers
who lit a candle of knowledge in my heart.

CONTENTS

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May God Almighty bless all of us and show the right path (Aamin).

FARHAT JABEEN

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LIST OF ABBREVATIONS

ACD	Acid citrate dextrose
ad	Autosomal Dominant
ar	Autosomal Recessive
CBL	Congenital Blindness
cM	centi Morgan
EBV	Epstein bar virus
EOG	Electro-oculogram
ERG	Electroretinogram
FCS	Fetal calf serum
GPPS	Glutamine pyruvate penicillin streptomycin
HBD	Homozygosity By Descent
IPC	Integral Plate Chamber
IPL	Inner Plexiform Layer
LCA	Leber's Congenital Amaurisis
LCL	Lymphoblastoid cell line
MD	Macular Degeneration
OPL	Outer Plexiform Layer
OS	Outer Segment
PBMC	Peripheral blood mononuclear cells
PDE	Phosphodiestrase
RDS	Rhodopsin
RP	Retinitis Pigmentosa
RPE	Retinal pigment epithelium
rpm	resolution per minute
SDS	Sodium Dodecyl Sulphate
SFD	Sorsby's Fundus Dystrophy
TEMED	N,N,N,N-tetra methyl ethylene diamine
TIMP3	Tissue inhibitor metalloproteinase-3
V/V	Volume by volume
W/V	Weight by volum

ABSTRACT

ABSTRACT

The present study includes five families affected with congenital blindness from different areas of Pakistan. The aim of the present study was to obtain a base line information regarding the development of eye disorders in some Pakistani families at molecular level. Clinical assessment was obtained before carrying out linkage analysis at molecular level in these families.

Family-1 was designated as 3CBL and was diagnosed for Leber's Congenital Amaurosis (LCA) with autosomal recessive mode of inheritance. The surname of the family was Abbasi. Their marriages were contracted both among cousins and distant relatives. The kindred spans on five generations. It consisted of 77 individuals out of which 62 were live and fifteen were dead at the time of study. Of these live members, thirteen members were affected including six females and seven males. Twenty nine individuals of the family were processed for molecular studies. These include 11 affected and 18 normal individuals. Of eleven blind individuals six were males and five females. The samples were further processed for genetic analysis at Biomedical and Genetic Engineering Division, KRL, Islamabad. Linkage analysis for known loci of autosomal recessive LCA loci was carried out. Significant linkage was found with LCA4 locus (17p13.1). Two point lod score analysis resulted in maximum lod score (Z max) of 4.75 for marker D17S796 at θ=0.

Family-2 was designated as 4CBL also diagnosed for Leber's Congenital Amaurosis (LCA) with autosomal recessive mode of inheritance. The surname of the family was Raja. The kindred spans on five generations. It consisted of 29 individuals out of which twelve were live and seventeen were dead at the time of study. Of these live members, five members were affected including two females and three males. For molecular studies peripheral blood was collected from seven individuals with informed consent of the family. These include four affected and three normal individuals. Linkage analysis for known loci of autosomal recessive LCA loci was carried out. Linkage was found with LCA4 locus (17p13.1). Two point lod score analysis resulted in maximum lod score (Z max) of 2.06 for marker D17S1832 at θ=0. This marker seems to be linked with disease region but due to less number of informative meioses this score is less than 3. And also the tightly linked marker for this region was unfortunately non-informative for this family.

Family-3 was designated as 7CBL and diagnosed for autosomal recessive macular degeneration. The surname of the family was Muslim Sheikh. Mostly consanguineous marriages were contracted within the kindred resulting in a higher number of affected individuals. The kindred spans on six generations and consists of 69 individuals, of which 48 were alive and 21 were dead at the time of study. Of these 48 live individuals 22 were affected, including 13 males and nine females. Twenty-eight members of this family were processed for molecular studies. As the individuals were congenitally blind initially known loci for LCA were excluded and later on known loci for retinal degeneration were searched. Linkage was found with microsatellite markers D6S1610, D6S1019 and D6S1017. Linkage studies revealed the presence of disease locus at 6p21.2.Two point lod score gave maximum value of 3.29 at $\theta=0$, for microsatellite marker D6S1019.

Family-4 was designated as 8CBL diagnosed for autosomal recessive hereditary fundus dystrophy. The surname of the family was Rajput. In this kindred mostly consanguineous marriages were contracted, resulting in a higher number of affected children. Of the live members, 12 were affected including five males and seven females. Homozygosity mapping revealed the presence of disease locus at chromosome 22q12.1-q13.2. Which is a previously reported locus for dominant Sorsby's fundus dystrophy. The microsatellite markers D22S280 and D22S685 showed hint of linkage with one branch through homozygosity mapping, while other two branches show homozygosity with different markers.

Family-5 was designated as 9CBL, diagnosed for autosomal recessive retinitis pigmentosa sine pigmento. The surname of the family was Arian. Mostly consanguineous marriages were contracted in the family resulted in a higher number of affected children.The kindred spans on five generations and consists of 45 individuals. Of which 27 were alive and eighteen were dead at the time of study. Of the live members, nine were affected including six males and three females. Nineteen family members were processed for molecular study. Of these 12 members were normal and seven were affected. Since in affected individuals night blindness was observed during first year of life, therefore, initially the affected members and their close degree relatives were screened to exclude the linkage to the regions of the previously described genes involved in LCA on chromosomes 1,6,14,17 and 19. During exclusion analysis, all the known loci for LCA were excluded. Then the

known loci of autosomal recessive Retinitis Pigmentosa (RP), autosomal recessive and autosomal dominant Cone-Rod dystrophies and autosomal dominant Retinitis Pigmentosa (RP), were also excluded and linkage was observed at $8q^{11}$-q^{13} with microsatellite markers, D8S285 and D8S1113. Two-point linkage analysis resulted in a maximum lod score of 3.11 for marker D8S285 at θ=0.

Present study revealed the presence of disease locus at LCA4 region (17p13.1) in 3CBL and 4CBL inbred pedigrees. We mapped the disease locus for autosomal recessive macular degeneration (7CBL) at 6p21.2 which is a previously reported locus for adMD and adRP from non-Asian populations (Dryja *et al.*1997, Kajiwara *et al.* 1994).. In the present pedigree this disease region shows maximum lod score of 3.29 at θ=0, with marker D17S1019 for autosomal recessive inheritance. These results suggest that inheritance for macular degeneration could be genetically heterogeneous (Michaelides *et al.* 2003a). Similarly, in family 9CBL it shows linkage at 8q11-q13 but this locus has been reported for adRP (Pierce *et al.* 1999; Jacobson *et al.*2000; Xu *et al.* 1996). In family 8CBL linkage may be shown in the disease region (22q13-qter2) due to homozygosity mapping because the family is highly inbred.

This study suggests that consanguineous marriages have a profound effect on the development of recessive eye disorders, and due to homozygosity autosomal recessive inheritance pattern is exhibited in all the families. A high percentage (60%) of consanguineous marriage has been observed in all the families studied here.

INTRODUCTION

GENERAL INTRODUCTION

Molecular genetics employs known principles of DNA structure and function to investigate the molecular basis for genotype-directed phenotypes under normal and pathological conditions. The DNA-multitalented molecule is the smallest unit of genetic heredity. Almost all organisms store their cellular blue prints for life in double stranded DNA. In Eukaryotic cells, chromosomes are copied during cell division, recombined and shuffled as a result of sexual reproduction, and transcribed into complementary RNA molecules through a process called, gene expression. This relationship between the DNA, RNA and protein sequence information of a gene is referred to as the flow of genetic information (Miesfeld, 1999).

The human genome is a double-stranded, linear polymer of DNA composed of approximately 3.3 billion nucleotide base pairs. The DNA sequences of these base pairs, a simple combination of four bases, adenine, thymine, guanine, and cytosine, encodes an estimated 50,000-100,000 genes and is tightly packed in 23 pairs of chromosomes (Winter *et al.* 1998). About 99% of the DNA sequence within the genome of any two individuals is identical (Guyer and Collins, 1993; Botstein *et al.*1980). The remaining 1% variability in the DNA sequences between any two individuals is responsible for a major portion of phenotypic variability (Guyer and Collins, 1993).

Genes that are present on the same chromosome are said to be linked and the tendency of parental recombinations to remain together is called linkage (Gardner *et al.*1991). However, during gamete formation, homologous chromosomes may recombine as a result of crossing over. The frequency of recombination depends on how close the two genes are located. If they are close to each other, there will be less recombination and more chances that these loci will coinherit in one chromosome, and are linked. In contrast, if two genes are far apart, or on different chromosomes, the frequency of recombination between them is greater and such loci are unlinked (Suzuki *et al.* 1989). The recombination fraction is a measure of distance separating two loci, or more precisely an indication of the likelihood that a cross over will occur between them; it is designated as θ (Muller and Young, 1995).

The unit of measurement for genetic linkage is known as a map unit or centi Morgan (cM). If two loci are one cM apart, then a crossover occurs between them during on average only 1 in every 100 meioses, i.e. $\theta = 0.01$ (Muller and Young, 1995).

The genetic map is the cornerstone of genetic linkage studies. Each gene has a locus, a specific location within a chromosome. Alternative forms of the same gene at its locus are termed alleles. The genes found in each chromosome have a specific order that is identical in all humans, with a few exceptions. Once a gene or marker is localized to a particular region on a chromosome, it is placed on a map. Therefore, the genetic map depicts the order of genes, genetic markers, and other landmarks that compose the chromosomes of a particular organism. There are two basic types of genetic maps: genetic linkage maps, which are based on the frequency with which genetic markers are coinherited, and physical maps, which represent the actual distance in base pairs between genetic markers or genes on a chromosome.

The human genome has been estimated by recombination studies to be about 3000 cM in length (Muller and Young, 1995). As the physical length of the haploid human genome is approximately 3×10^9 base pairs, 1cM corresponds to approximately 10^6 base pairs (or 1000 kilobases). However, the relationship between linkage map units and physical length is not linear. Some chromosome regions appear to be particularly prone to recombination, so-called 'hot spots', and for reasons, which are not understood, recombination tends to occur more often during meiosis in the female than male. As a rough idea, during meiosis I large chromosomes would be expected to show an average of three crossovers, medium sized chromosomes two crossovers and small chromosomes one crossover (Muller and Young, 1995)

An essential tool for performing genetic linkage analysis successfully is a group of genetic markers that have multiple alleles (that is, are polymorphic) and have a known locus. Markers are inherited in the same manner as genes (the mendilian pattern) and therefore, can be traced through pedigrees. The DNA sequence of each marker must be highly variable so that its maternal or paternal origin can be determined. When this is the case, the marker is considered useful or informative for linkage analysis. The more alleles a marker has, the more likely it will be that the maternal and paternal genotypes will

differ and, therefore, that the paternal origin of a specific allele can be determined in the children (Damji and Allingham, 1997).

When the inheritance pattern of a disorder is known (for example, the pattern is dominant, recessive or X-linked), a model-depedent analysis is used, which is called lod score (Elston, 1995). If the inheritance pattern is unknown, (for example, complex disorder), a model independent method is used, for example, a sibpair or affected pedigree member method (Elston, 1995). Evidence for linkage includes a lod score of +3 or more or a significant statistic in model independent methods. It is also important to obtain independent confirmation of linkage examining a completely separate data set (that is, different families) or by having another investigator replicate the findings. This is especially important for complex diseases (Lander and Schork, 1994).

Linkage Analysis:

Genetic linkage analysis is the technologic powerhouse and often the critical first step in identifying a disease-causing gene. The essence of this approach is to compare, within a family, the inheritance of a disease gene with the inheritance of specific DNA segments termed as markers. Coinheritance of the disease gene and marker suggests that they are physically close, that is, linked on a specific chromosome. Linkage analysis provides an approximate position of the disease gene within the genome. Fine genetic mapping can further refine this location. Eventually the gene is cloned and sequenced. Mutations can be identified and the role of the gene in the pathophysiology of the disease studied (Damji and Allingham, 1997). After the coinheritance of the disease gene and the marker, Lod scores are calculated. Once genes that cause inherited disorders are identified and sequenced, disorders not only can be understood but also can be treated at the molecular level (Hanania *et al.* 1995).

LOD score (Z):

Lod scores are the statistical measures of the evidence for linkage (Strachan, 1992; Strachan and Read, 1996; Strachan and Read, 2000). The Lod score, Z, introduced by Morton (1995) is the logarithm of the odds that are linked (with recombination fraction θ) rather than unlinked (with recombination fraction 0.5). Lod scores are calculated for a range of θ values and the maximum value of Z is estimated (Strachan,

1992; Strachan and Read, 1996; Strachan and Read, 2000). By convention, a positive lod score of 3.0 or more is accepted as proof of linkage (1,000:1 odd favouring linkage versus no linkage), whereas a negative score of –2 or less is evidence against linkage (100:1 odds of no linkage versus true linkage). Values of lod score between –2 and +3 are inconclusive. Several factors can influence the lod score, such as the certainty of clinical diagnosis, the size of the family being examined, the number of affected individuals and their location within the pedigree, the mode of inheritance, and the informativeness of the genetic markers used (Damji and Allingham, 1997).

Homozygosity mapping/Gene localization:

Homozygosity mapping is a powerful strategy for mapping rare recessive traits in children of consanguineous marriages. Rare recessive traits appear in children of consanguineous marriages more often than in the general population. The appearance of such traits in inbred children usually is due to homozygosity by descent (HBD) for a single disease-causing allele inherited from a recent ancestor common to both the maternal and paternal lineage. This not only explains the high incidence of such traits in consanguineous marriages but also provided a powerful tool for genetic mapping of the disease causing genes. Homozygosity mapping involves locating a gene causing rare recessive trait by using multipoint linkage analysis to find regions of HBD shared among inbred affected children. The method does not require the availability of families with multiple affected individuals but it can utilize unrelated affected members from different consanguineous marriages. In principle, three offspring from a first cousin marriage are sufficient to obtain a LOD score of 3.0 (odds of lineage are >1000:1). Homozygosity mapping is well suited to a wide variety of recessive traits of medical or biological interest for which it is impractical or impossible to gather a large collection of multiPlex families (Guilford *et al.* 1994).

Eye:

The human eye is a complicated organ, comprising a number of different tissue types that are derived from all three embryological layers. It is not surprising, therefore, that the eye is one of the commonest sites of genetic disease. The importance of this

group of disorders is also reflected in the simple fact that genetic eye diseases, both monogenic and genetically complex, comprise the commonest causes of blindness in children and adults in the developed world (Gregory-Evans and Bhattachararya, 1998).

The human eye is wrapped in three layers of tissue (www.webvision/vision 1.html 2 August, 2003) (Fig. 1):

1. The external layer is formed of sclera and cornea.

- **Sclera** is the white part of the eye, a tough covering with which the cornea forms the external protective coat of the eye.
- **Cornea** is the transparent circular part of the front of the eyeball. It refracts the light entering the eye onto the lens, which then focuses it onto the retina. The cornea contains no blood vessels and is extremely sensitive to pain.

2. The intermediate layer is divided into two parts: anterior (iris and ciliary body) and posterior (choroid).

- **Iris** regulates the amount of light that enters the eye. It forms the coloured, visible part of the eye in front of the lens. Light enters through a central opening called the pupil.
- **Ciliary body** is the part of the eye that connects the choroid to the iris.
- **Choroid** is the middle layer of the eye between the retina and the sclera. It also contains a pigment that absorbs excess light so preventing blurring of vision.

  LENS is a transparent structure situated behind the pupil of the eye and it is enclosed in a thin transparent capsule. It helps to refract incoming light and focus it onto the retina.

3. The internal layer, or the sensory part of the eye (the retina).

- **RETINA:**

Retina is a light sensitive layer that lines the interior of the eye. It is composed of light sensitive cells known as rods and cones. The human eye contains about 125 million rods, which are necessary for seeing in dim light. Cones on the other hand function best in bright light and are about between 6 and 7 million in the eye. Cones are essential for receiving a sharp accurate image and can also distinguish colors.

Macula is a yellow spot on the retina at the back of the eye, which surrounds the fovea. This is the area with the greatest concentration of cone cells.

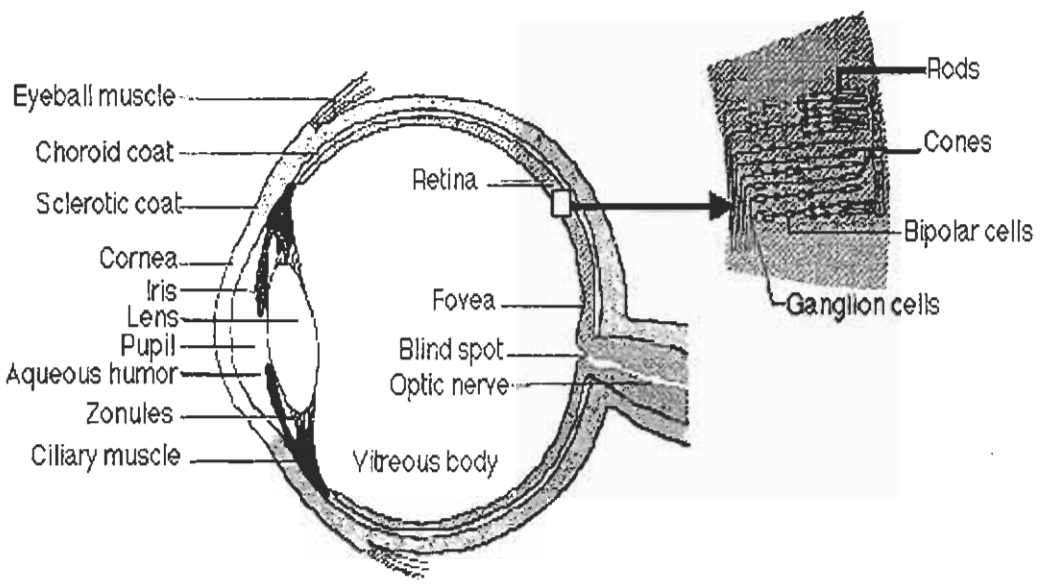


Fig.1 Anatomy of Human Eye.

Fovea forms a small indentation at the centre of the macula and is described as the area with the greatest concentration of cone cells.

Optic disk is the visible (when the eye is examined) portion of the optic nerve also found on the retina of the eye. The optic disk identifies the start of the optic nerve where messages from cone and rod cells leave the eye via nerve fibres to the optic centre of the brain. This area is also known as the 'blind spot'.

Optic nerve leaves the eye at the optic disk, and transfers all the visual information to the brain.

Eye chambers:

Eye also consists of three chambers (Fig.1). Anterior chamber (between cornea and iris), Posterior chamber (between iris, zonule fibers and lens) and the Vitreous chamber (between the lens and the retina). The first two chambers are filled with aqueous humor whereas the vitreous chamber is filled with a more viscous fluid, the vitreous humor.

Extraoccular muscles:

Each eyeball is held in position in the orbital cavity by various ligaments, muscles and fascial expansions that surround it.

RETINAL ORGANISATION:

The retina is approximately 0.2 mm thick, and has an area of approximately 1100 mm^2 (about the size of a silver dollar) and lines the back of the eye. The optic nerve contains the ganglion cell axons running to the brain and, additionally, incoming blood vessels that open into the retina to vascularize the retinal layers and neurons (Fig. 2). When an anatomist takes a vertical section of the retina and processes it for microscopic examination (Fig.3), it becomes obvious that the retina is much more complex and contains many more nerve cell types than the simplistic scheme (Fig.1 and 2) had indicated. It is immediately obvious that there are many interneurons packed into central part of the section of retina intervening between the photoreceptors and the ganglion cells (Hagerman and Johnson, 1991; Henkind *et al.* 1979; Kolb, 1991). All vertebrate retinas are composed of three layers of nerve cell bodies and two layers of synapses. The outer nuclear layer contains cell bodies of the rods and cones, the inner nuclear layer contains cell bodies of the bipolar, horizontal and amacrine cells and the ganglion cell layer contains cell bodies of ganglion cells and displaced amacrine cells. Dividing these nerve

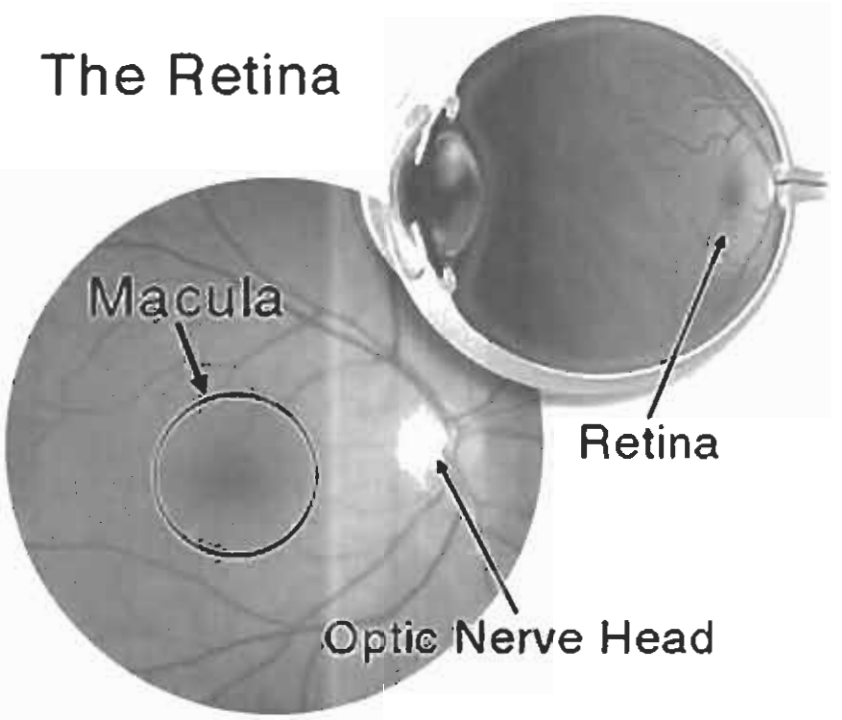


Fig.2: The Human Retina (Adapted from illustration by Mark Erickson (http://www.stlukeseye.com/Anatomy.asp).

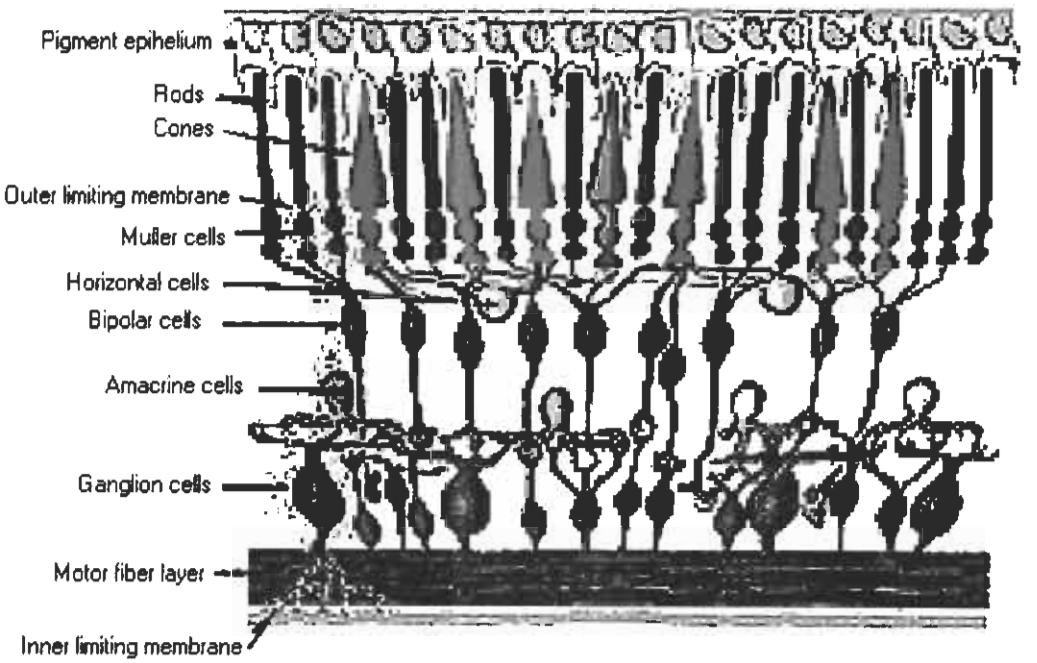


Fig.3: Simple organisation of the retina

cell layers are two neuropils where synaptic contacts occur.

The first area of neuropil is the outer plexiform layer (OPL) where connections between rod and cones, and vertically running bipolar cells and horizontally oriented horizontal cells occur.

The second neuropil of the retina, is the inner plexiform layer (IPL), and it functions as a relay station for the vertical information carrying nerve cells, the bipolar cells, to connect to ganglion cells. In addition, different varieties of horizontally and vertically-directed amacrine cells, somehow interact in further networks to influence and integrate the ganglion cell signals. It is at the culmination of all this neural processing in the inner plexiform layer that the message concerning the visual image is transmitted to the brain along the optic nerve.

Phototransduction and Visual Cascade:

Phototransduction and the visual cycle play complementary roles in human vision. Vision begins in the outer segments (OS) of rod (dim light) and cone (bright) photoreceptor cells of the retina where visual pigment molecules absorb photons and become activated. Individual activated pigment molecules carry the molecular activation signal to G proteins, which in turn activate the phosphodiesterase (PDE) enzyme to break down aqueous cyclic GMP (cGMP). This causes cGMP to be released from the plasma membrane ion channels where its binding sustains the inward flowing cation current that keeps visual cells depolarized in darkness (Chabre and Deterre, 1989). The depolarization, as in other nerve cells, causes release of a neurotransmitter from the synaptic terminals of photoreceptors. The neurotransmitter signals other cells of the retina, a neural network those microprocesses visual images before sending the result to the brain via the optic nerve. Thus, light causes rod and cone synaptic transmission to be terminated by activating a chain of molecules that result in closure of the ion channels that conduct a "darkness" action current. Sight is surprisingly caused by termination of background activity by light. Indeed, following illumination, the entry of Ca^{2+} through the cGMP-gated channel stops but its export by the exchanger continues. Consequently, the cystolic level of Ca^{2+} drops (from about 500 nM to 50nM), stimulating the production of guanylate cyclase (Dizhoor *et al.* 1994). In the recovery process of photoreceptors, the photo-activated transduction cascade is abolished and the level of cGMP concentration in

photoreceptors is restored to the dark level by the conversion of GTP to 3', 5' cGMP catalysed guanylate cyclase (Fig.4). Newly synthesized cGMP reopens the channels to restore the dark state (Lolley and Lee, 1990), and Ca^{2+} as well as Na^{+} enter the photoreceptor outer segment, through cGMP-gated channels.

Mutations in genes encoding the components of phototransduction cascade i.e; rhodopsin, the tranducin ∝ subunit, the catalytic PDE subunit, the CNG channel subunits, and the GC1/GCAP1 modulatory unit have been shown to cause autosomal dominant or recessive retinal degenerations (Andrew *et al.* 2001) .

Diseases of retina.

The following diseases of the retina fall into two major categories:

Normal Appearing Retina (Non-progressive Diseases):

These conditions have characteristics, which include: bilateral decreased vision; purposeless, involuntary, rhythmic eye movements (nystagmus); and an apparently normal clinical examination; however, an abnormal ERG establishes the appropriate diagnosis (Lambert and Taylor, 1989). These include:

- **Lebers Congenital Amaurosis (LCA-also known as Cone/Rod Dystrophy)**:

A group of disorders with little or no vision, slow nystagmus-like movements, abnormal amounts of farsightedness (3 diopters or more), and an extinguished (flat) ERG. Most of the children will have no other associated findings. However, some will be found to have kidney, brain or heart disorders, for example: Senior- Loken Syndrome or Joubert's Syndrome.

- **Cone Dystrophies**: Usually present with mild to moderate vision loss, photophobia and small amplitude nystagmus. Color vision is variably impaired.

- **Achromatopsia**: The most common of the cone disorders presenting in infancy with reduced visual function, color blindness, light sensitivity (photophobia) and a fast.moving "shimmy" of the eye (nystagmus). Tinted lenses improve visual function. This is an autosomal recessive condition with no associated abnormalities.

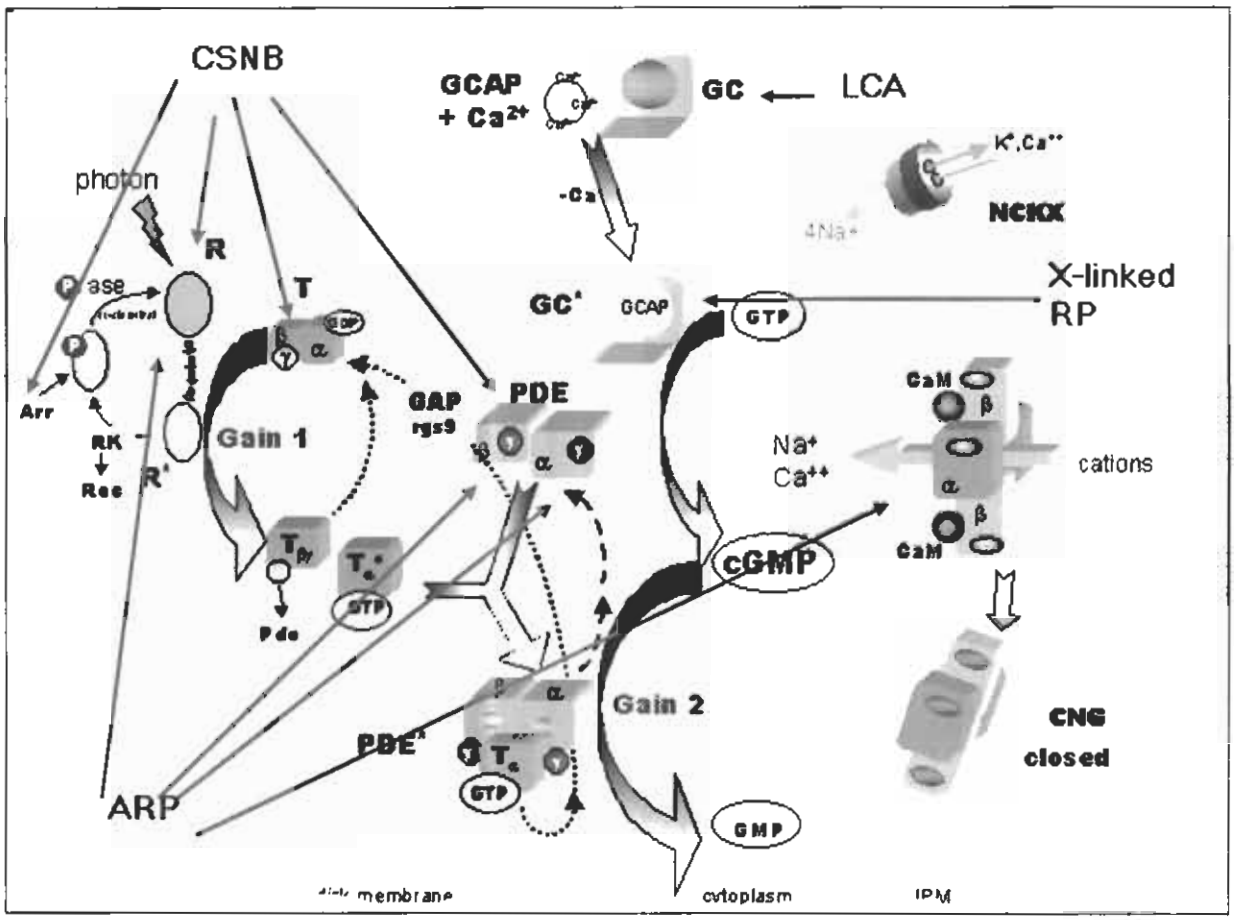


Figure 4. The phototransduction cascade shows the pathway by which light energy is converted to chemical energy with in a photoreceptor. Rhodopsin serially activates many copies of the G protein, transducin (G_t) (gain 1), and the α subunit of G_t, in turn, activates a cGMP-specific phosphodiesterase (PDE), which degrades cytoplasmic cGMP with rapid turnover (gain 2). The drop in [cGMP] causes cGMP-gated cation (CGC) channels in the plasma membrane to close, which produces hyperpolarization of the outer segment plasma membrane. Closure of cation channels prevents entry of cations, and cytoplasmic [Ca^{2+}] drops due to its continued extrusion by the light-independent exchanger, also located in the plasma membrane. In a negative feedback loop, the drop in [Ca^{2+}] causes stimulation of a guanylate cyclase (GC) by one or several specific Ca^{2+} binding proteins, termed guanylate cyclase activating proteins (GCAPs). Sites where regulatory proteins (Phosducin, Pdc; arrestin, Arr; recoverin, Rec) interact with components of the cascade are indicated by arrows. Each of the proteins in this cascade is a potential candidate gene for retinal degeneration because of its critical role with in the photoreceptor. Pink arrows indicate that ARP (autosomal retinititis pigmentosa), X-linked RP, LCA (Leber's congenital amaurosis), and Congenital stationary blindness is caused in the mutation of genes involved in visual cascade(Adapted from a drawing by Dr. Wolfgang Baehr;http://insight.med.utah.edu/research/basic_science/baehr/baehr_lab.htm).

- **Congenital Stationary Night Blindness**: A rod dystrophy occurring only in boys, who present with night blindness, nystagmus, decreased visual function and near sightedness (myopia). This is an X-linked recessive inheritance with no associated abnormalities.

2. Abnormal Appearing Retina

These conditions fall into three categories:

a. **Congenital and Acquired Structural Abnormalities**
b. **Traumatic Retinal Detachment/Hemorrhage**
c. **Infections**

a. Congenital and Acquired Structural Abnormalities

Children with structural abnormalities of the retina present with a wide variety of syndromes and conditions.

- **Colobomas**: Congenital absence of retinal tissue (cleft or hole) caused by failure of normal development in the sixth week of pregnancy. Visual function is dependent on the size and location of the cleft.

- **Vitreoretinal Dysplasia (Norries disease)**: Congenital disorganization of the normal structure of the vitreous and retina, which usually causes blindness. This is an X-linked recessive form of vitreoretinal dysplasia associated with retardation and deafness.

- **Sticklers Syndrome**: Autosomal dominant disorder, leading to cataracts, retinal detachment and glaucoma. These children have high myopia, which is generally non-progressive. Careful monitoring of children with Sticklers is important because early treatment of ocular complications can prevent blindness.

- **Retinitis Pigmentosa (RP a rod/cone dystrophy)**: Progressive, hereditary degeneration and wasting away (atrophy) of light sensitive cells (rods and cones) of the retina, with differing rates of progression and severity, and different modes of

inheritance. RP begins with rod dysfunction only; but as the disease progresses, the cones are involved as well. RP does not present with acuity and color vision loss in early childhood, but caregivers frequently notice that children exhibit reduced peripheral vision and night blindness. RP affects approximately 1 in 4000 persons. Deafness is sometimes associated with RP, usually occurring later in life.

- **Retinoblastoma**: Malignant tumor within the eye, usually presenting before age 5, with one crossed eye (monocular strabismus) or a white spot or glow in the pupil (abnormal light reflex). Retinoblastoma can be hereditary or non-hereditary affecting one or both eyes (unilateral or bilateral).

b. Traumatic Retinal Detachment/Hemorrhage:

When detached from its normal blood supply, the retina can no longer maintain its normal activity and function.

- **Shaken Baby Syndrome (SBS):** When an infant is forcefully shaken, detachment of the retina may occur as either the result of direct traumatic injury or as a secondary complication of intraocular bleeding. The visual impairment may be due to retinal detachment, optic atrophy, and/or damage to visual pathways in the brain.

- **Head Injury**: A similar destructive injury, most commonly occurring in auto accidents.

C. Infections

- **Toxoplasmosis**: Infection of the eye occurring as a result of maternal exposure (during pregnancy) to a parasite present in cat feces. The brain may also be affected.

- **Toxocara**: An acute intraocular inflammatory response to a parasite found in dog feces. The parasite causes cataracts, inflammation of the vitreous and surrounding tissues (vitritis).

- **Herpes**: A blood-born viral infection transmitted during pregnancy and/or delivery that may cause a devastating destruction (necrosis) of the retina and brain.

- **Cytomegalovirus (CMV)**: A viral infection transmitted during pregnancy that may

cause damage to the retina, brain and liver.

Genetics of Retinal Degenerations:

Humans are afflicted with a large and heterogenous group of inherited blinding diseases. Most share the common histopathological feature of photoreceptor-cell death. The prototypical disease in this group is retinitis pigmentosa (RP), with the clinical phenotype of progressive night blindness and tunnel vision, advancing to complete visual loss in later life. As a group, human retinal degenerations are characterized by both allelic and nonallelic heterogeneity. For example, mutations in multiple unrelated genes may cause the RP phenotype, whereas different alleles of a single gene, such as *RDS,* may cause clinically disparate retinal diseases.

Many genes have been identified as responsible for human retinal degeneration. The genes for several proteins in the visual transduction pathway have been implicated, including those for rhodopsin (Rao and Oprian, 1996; Shastry, 1997), transducin (Dryja *et al.* 1996), both α- and β-catalytic subunits of cGMP-phosphodiesterase (PDE) (Huang *et al.* 1995; McLaughlin *et al.* 1995), the cGMP-gated cation channel (Dryja *et al.* 1995), rhodopsin kinase (Yamamoto *et al.* 1997), arrestin (Fuchs *et al.* 1995), and guanylate cyclase (Perrault *et al.* 1996). Mutations in genes for other photoreceptor-specific proteins, including rds/peripherin (Shastry, 1997), rom1 (Kajiwara *et al.* 1994), rim protein (RmP) (Allikmets, 1997; Allikmets *et al.* 1997; Azarian and Travis, 1997), and crx, a newly discovered otx-like homeodomain protein (Freund *et al.* 1997), have been reported to cause retinal degeneration. Photoreceptor-cell death also may be caused by mutations in genes expressed in the overlying retinal pigment epithelium (RPE), including the genes for cellular retinaldehyde binding protein (CRALBP) (Maw *et al.* 1997) and RPE65 (Gu *et al.* 1997; Marlhens *et al.* 1997).

Finally, retinal degeneration, which, in some cases, is a component of a more complex disease phenotype, may be caused by mutations in widely expressed genes, including those for myosin VIIA (Weil *et al.* 1995), rab geranylgeranyl transferase (Seabra *et al.* 1993), the cytosolic retinitis pigmentosa GTPase regulator (Meindl *et al.* 1996), and the tissue inhibitor of metalloproteinase-3 (TIMP3) (Weber *et al.* 1994).

Despite the large number of identified loci, the molecular defect cannot be found in >50% of patients with RP only. Since commonly affected genes generally are discovered early, the total number of genes responsible for human inherited retinal degeneration may be several-fold greater than the number identified to date. This number of mutations affects no other mammalian-cell type.

The Apoptotic Pathway of Photoreceptor Degeneration:

Little is known about the mechanism of photoreceptor-cell death in humans, owing to the scarcity of surgical or autopsy specimens of retina from RP patients with defined genetic lesions. However, animals that carry spontaneous or engineered mutations in multiple genes implicated in human inherited retinal degeneration have been described. The mode of cell death in several of these animal systems has been studied, including in (1) the *retinal degeneration* (*rd*) mouse (Chang *et al.* 1993; Lolley *et al.* 1994; Portera-Cailliau *et al.* 1994); (2) the *retinal degeneration slow* (*rds*) mouse (Chang *et al.* 1993; Portera-Cailliau *et al.* 1994); (3) transgenic mice carrying the RP-associated mutations in the rhodopsin gene, P347S and Q344ter (Chang *et al.* 1993; Portera-Cailliau *et al.* 1994); (4) knockout mice deficient for the β2-subunit of Na^+/K^+-ATPase expressed in retinal Müller cells (Molthagen *et al.* 1996); (5) Royal College of Surgeons (RCS) rats (Tso *et al.* 1994); (6) albino rats undergoing light-induced photoreceptor degeneration (Abler *et al.* 1996); and (7) cats with experimental retinal detachments (Cook *et al.* 1995). In every case, photoreceptors were shown to die via the apoptotic pathway, as evidenced by the histological picture, by terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick end-labeling assays, and/or by direct demonstration of retinal-DNA nucleosomal laddering by gel electrophoresis. On the basis of these studies, it is likely that, in most if not all forms of human retinal degeneration, photoreceptors similarly are dying by apoptosis. Unfortunately, this statement says little about what is actually killing the cells.

Genetics of Blindness:

Blindness is a very common neurosensory defect but it is not a reportable disease, however, it is estimated that at least 50% of new cases of legal blindness (i.e.20/200) or less than 20 degrees of peripheral vision) can be attributed to genetic cause (Nelson *et al.* 1991).

Mapping genes causing blindness is of vital importance. About 1000 inherited eye diseases affect both the anterior compartment comprising of cornea, iris and lens, and the posterior comprising of viterous and retina. Hereditary causes are identified in up to 50% of the visually handicapped individuals. In them most common and widespread are retinal disorders, which account for 15 to 55%.

Table-1 shows expression of previously mapped and cloned retinal disease gene (http://www.sph.uth.tmc.edu/Retnet/disease.htm). Retinal disorders may be congenital, inborn or acquired (Scott, 1997). A variety of systemic and ocular diseases result in the degeneration of retina characterized by loss of visual receptors accompanied by focal proliferation of the adjacent retinal pigment cells into sensory retina. In humans, the retinal degeneration progresses slowly often taking decades. In contrast, animal models reproduce the degenerative disease during a course of several weeks to several months (Nash *et al.* 1993; Nishikawa *et al.* 1997; Olsson *et al.* 1992).

The hereditary retinal degeneration tend to be more severe that lead from little vision loss to complete blindness. There are at least 200 inherited diseases that lead to retinal degeneration in humans. Table-2 summarizes the genes causing retinal diseases by genes and diseases (http://www.sph.uth.tmc.edu/Retnet/disease.htm).

EPIDEMIOLOGY OF CHILDHOOD BLINDNESS:

Prevalence of childhood blindness:

There are few sources of population based data on the prevalence of childhood blindness and low vision and no sources of data on their incidence. In industrialized countries, some information is available from blindness registries, and population-based studies of adult blindness in developing countries have given some crude estimates of the prevalence of blindness in children. In general terms the prevalence of blindness is at least three to five times greater in poor areas of the world than in industrialized countries. It is estimated that there are approximately 1.5 million blind children in the world (Table-3), and an estimated 500000 become blind each year, of whom probably more than half die in childhood (WHO, 1997; Foster and Gilbert, 1997,1992). The prevalence of low vision is probably three to four times greater than that of blindness, with approximately 5 million children being affected worldwide. Retinal photoreceptor

Table 1. Expression of previously mapped and cloned retinal disease genes.

Summary of retinal disease genes	**Total genes**
Retinal disease genes mapped	124
Retinal disese genes cloned	71
Expression pattern of cloned disease genes	
Rod-specific	26
Rod-enriched	7
Cone-specific	5
RPE-specific	6
Rod and RPE-enriched	2
Enriched in retina/cellular localization unknown	3
Widespread	19
Non-retinal	1
Retinal progenitors	2
Total	71
Total rod-specific+rod-enriched	33

Table-2. Summary of Genes Causing Retinal Diseases By genes and Diseases.

Disease Category	Total No. of Genes	No. of Cloned Genes
Bardet-Biedl syndrome, autosomal recessive	8	6
Cone or cone-rod dystrophy, autosomal dominant	6	4
Cone or cone-rod dystrophy, autosomal recessive	2	0
Cone or cone-rod dystrophy, X-linked	2	0
Congenital stationary night blindness, autosomal dominant	1	1
Congenital stationary night blindness, autosomal recessive	2	2
Congenital stationary night blindness, X-linked	2	2
Leber congenital amaurosis, autosomal recessive	7	4
Macular degeneration, autosomal dominant	11	6
Macular degeneration, autosomal recessive	1	1
Ocular-retinal developmental disease, autosomal dominant	1	0
Optic atrophy, autosomal	2	1

dominant		
Optic atrophy, autosomal recessive	1	0
Optic atrophy, X-linked	1	0
Retinitis pigmentosa, autosomal dominant	12	11
Retinitis pigmentosa, autosomal recessive	15	11
Retinitis pigmentosa, X-linked	5	2
Syndromic/systemic diseases with retinopathy, autosomal dominant	7	5
Syndromic/systemic diseases with retinopathy, autosomal recessive	14	11
Syndromic/systemic diseases with retinopathy, X-linked	2	1
Usher syndrome, autosomal recessive	11	7
Other retinopathy, autosomal dominant	8	3
Other retinopathy, autosomal recessive	15	11
Other retinopathy, mitochondrial	6	6
Other retinopathy, X-linked	9	7
TOTALS	**151**	**102**

Table-3. Estimated magnitude of childhood blindness by region.

Region	Population <16 years of age (millions)	Blindness prevalence(per 1000 children)	Estimated number of blind children
Africa	240	1.1	264000
Asia	1200	0.9	1080000
Central and South America	130	0.6	78000
Europe/Japan/ USA	240	0.3	72000
TOTAL	1810		1494000

dystrophies are a clinically and genetically heterogeneous group of retinal degenerations that together form the most frequent cause of inherited visual disorders, with an estimated prevalence of 1 in 3500 (Sullivan *et al.* 1999). It is present in three million young adults throughout the world and in about 150000 of those in Pakistan (Denton, 1998). Stephen P. Daiger, PhD., Professor of Human Genetics Center, School of Public Health and Dept. of Ophthalmology and Visual Science, The University of Texas – Houston, reported the classification of all major and sub-categories of inherited retinal diseases and their prevalence (www.mdsupport.org). He reported a summary based on published surveys, clinical reports and educated guesses.

The numbers are based on the American population, and surveys in Europe, but they also apply throughout the world, because these conditions affect all groups of people roughly the same. Table-4 and 5 represent the broad categories and sub-categories of inherited retinal diseases.

Causes of childhood blindness:

According to WHO there are two distinct ways of classifying causes of childhood blindness and low vision (Foster and Gilbert, 1997,1992). The first is a descriptive, anatomical classification according to the level at which vision is obstructed in the eye (Table-6). The second classification is by etiology, according to the developmental time at which the insult has occurred (Table-7). Most of the information on the causes of childhood blindness and low vision has come from the examination of children in schools for the blind. For various reasons, these results may not be representative of the total population of visually impaired children, as the schools tend to be in cities and usually admit children from older age groups, so that preschool children, children from rural areas and also those with additional disabilities may not be adequately represented. The main causes of childhood blindness in developing countries are preventable and include conditions that give rise to corneal scarring, e.g. vitamin A deficiency, measles, harmful traditional eye practice, ophthalmia neonatorum and other corneal infections. Potentially treatable conditions include cataract and glaucoma. In Industrialized countries, the main causes of blindness are lesions of the central nervous system and hereditary diseases (particularly affecting the retina), some of that may be amenable to preventive measures such as genetic counselling. Retinopathy of prematurity, which is a potentially avoidable

Table-4. Broad categories of inherited retinal diseases.

Sr.No.	Retinal diseases	%
1	Retinitis pigmentosa	40
2	Usher syndrome	10
3	Cone-rod dystrophy	10
4	Inherited macular degeneration	10
5	Chorioretinal degeneration	5
6	Leber congenital amaurosis	5
7	Congenital stationary night blindness	5
8	Retinoschesis	3
9	Choroideremia	2
10	Other inherited retinopathies	10
	TOTAL	100

Table-5. Sub-categories of inherited retinal diseases.

1	Retinitis pigmentosa (40% of total)	%
a	Autosomal dominant	10
b	Autosomal recessive	8
c	X-linked	6
d	Single case or unknown	16
2	Usher syndrome (10%) -- nearly all recessive	10
3	Cone-rod dystrophy (10%)	
a	Autosomal dominant	2
b	Autosomal recessive	2
c	Other/unknown	6
4	Inherited macular degeneration. (10%)	
a	Autosomal dominant	2
b	Autosomal recessive	1
c	Other/unknown	7
5	Chorioretinal degeneration (5%) -- nearly all recessive	5
6	Leber congenital amaurosis (5%) -- nearly all recessive	5
7	Congenital stationary n.b. (5%)	
a	Autosomal dominant	1
b	Autosomal recessive	1
c	X-linked	1
d	Other/unknown	2
8	Retinoschesis (3%) -- nearly all X-linked	3
9	Choroideremia (2%) -- nearly all X-linked	2
10	Other inherited retinopathies (10%) -- unknown mixture	10

Table-6 Anatomical classification of causes of childhood blindness and low vision.

Whole globe	e.g. microphthalmos, anophthalmos, phthisis bulbi
Cornea	Corneal scarring, anterior staphyloma, dystrophies
Lens	Cataract, dislocated lens
Uvea	Aniridia, chronic uveitis, coloboma
Retina	Retinopathy of prematurity, retinal dystrophies, retinal detachment
Glaucoma	buphthalmos
Optic nerve	Optic atrophy, optic nerve hypoplasia
Other	Cortical blindness, amblyopia

Table-7 Etiological classification of causes of childhood blindness and low vision.

Hereditary	Autosomal dominant or recessive disease,X-linked,mitochondrial and chromosomal abnormalities
Intrauterine	Congenitally acquired rubella, fetal alcohol syndrome
Perinatal	Ophthalmia neonatorum, retinopathy of prematurity
Childhood	Vitamin A deficiency, measles, harmful traditional eye practices, trauma.
Unclassified	Impossible to determine the underlying cause

cause of childhood blindness, is important where very-low birth-weight babies (less than 1500g) survive. Congenital cataract and congenital glaucoma together represent 10-20% of childhood blindness in most parts of the world.

Objective of the study:

It was intended to carry out studies both at epidemiological and molecular level on families with congenital blindness. The study would help in the ascertainment of inheritance and location of gene on autosomal chromosomes. The main aim was to gather clinical explanation and to carry out linkage analysis at molecular level in Pakistani families. It is expected that study would be interesting at molecular level to see how far consanguinity in these families enhances the appearance of the disease because of homozygosity.

MATERIALS AND METHODS

MATERIALS AND METHODS

The families for epidemiological and molecular studies were collected from Islamabad and Rawalpindi district, District Faisalabad, District Mianwali, and District Jhang. Five families indicated necessary relevant information for molecular studies, which were proceeded further for studies at molecular level. The criteria for molecular studies observed in this study are as follows:

INCLUDING CRITERIA

- Family at least consists of 5 generations.
- Family should be cooperative in behavior.
- At least 5 individuals should be afflicted with congenital blindness in the family, to get better results both through clinical examination and at molecular level.
- Family should have at least 11 informative meioses, to calculate lod score.
- It should not be sampled earlier by some on else.

The details of the family were traced back through the proband. The information collected include the type of marriage, exact relationship between husband and wife, parental relationship of parents of husband and wife, their family history including information about number of offspring (sex, birth order), similar disease and other disease in family, surname of the patients, normal and diseased individuals in the family, and age of onset was also recorded. Information about economic status and educational qualification of couples or the parents (as the case may be) was also recorded. The clinical assessment was done by an ophthalmologist, and involvement of other organs was also recorded.

Pedigrees were drawn by using the software package, Cyrillic (version 2.1).

Molecular Studies:

At molecular level the families were processed at Biomedical and Genetics Engineering (B&GE) Division, KRL hospital, Islamabad.

Blood Sample Collection:

Peripheral blood samples were collected with informed consent from the affected and normal members of the families. Seven to ten milliliter (ml) of the blood was

collected in vaccutainer tubes (Becton Dickinson, Franklin Lakes, and NJ) containing Acid Citrate Dextrose (ACD) and were maintained at room temperature for 24-72 hours.

Isolation of Peripheral Blood Lymphocytes:

10 ml of blood was layered over 5ml Ficoll (Histopaque-1077) in a 15 ml sterile Falcon tube and centrifuged at 2000 rpm for 25 minutes. Buffy coat containing peripheral blood mononuclear cells (PBMC) were collected from the interface of Ficoll into a new tube and washed twice with RPMI-1640 containing 1% Glutamine Pyruvate Penicillin Streptomycin (GPPS) and 1% Fetal calf Serum (FCS). After centrifugation at 1000 rpm, for 10 minutes, the supernatant was discarded and the cell pellet was washed with 5 ml of washing media for 10 minutes at 1000 rpm. Then supernatant was discarded and cell pellet was resuspended in 1ml of the washing medium.

Transformation With Epstein Bar Virus (EBV):

Walls and Crawford (1987) method was used for transformation with Epstein Bar Virus (EBV). EBV is a double stranded DNA virus that infects human B- lymphocytes. It enters the lymphocytes through CD23 receptors and transforms them into continuously growing lymphoblastoid cell lines, which can provide a constant source of DNA. Approximately 2-3 x 10^6 lymphocytes were transferred to a $25cm^3$ culture flask containing 3ml of transformation medium (RPMI-1640 supplemented with 1% GPPS (Glutamine Pyruvate Penicillin Strepto -mycin), 10% FCS (Fetal Calf Serum), $5x10^{-5}$ M β-mercaptoethanol and 0.5 ug / ml cyclosporin A). EBV supernatant (1ml) was added to the culture flask and cells were incubated at 37 C^0 in a humified atmosphere of 95% air and 5% CO_2 (Walls and Crawford, 1987). The flask caps were tightened after 24 hours. Depending on the initial number of lymphocytes, EBV immortalized cells could be visualized after 1-4 weeks of culture as characteristic free floating round clumps. Increased acidity of the medium was an additional sign of establishment of lymphoblastoid cell lines (LCL). The transformed cells were periodically examined using an inverted microscope and the cell cultures were fed with feeding media. Once the cell lines established they were split into $75cm^3$ flask and expanded for cryogenic preservation and DNA preparation.

Cell Viability Test:

Cell viability was measured by Trypan Blue exclusion test (Hunt, 1987) using a heamocytometer (Neusauer). The number of live and dead cells was counted in 25 squares in the center of the counting chamber of the heamocytometer and the percentage viability was calculated by the following formula:

Percentage Viability= Number of live cells /total number of cells x 100

The number of live cells per ml was calculated by the following formula:

Live cell count x dilution factor (2.0) x 10^4

Cryopreservation and Revival of Frozen Culture:

After determining that cell viability of each culture was 90% or greater approximately 5 x10^6 cells were frozen per vial for each culture. Cells were pelleted by centrifugation at 1000 rpm for 10 minutes and the supernatant was aspirated. The pellet was resuspended in 1 ml of freezing medium which consisted of 10 % DMSO (Dimethyl Sulphoxide), 45% RPMI-1640 and 45% FCS. Four vials were frozen for each culture, first each was stored at –70C^0 and then transeferred in liquid nitrogen. When required, the vials containing frozen cells were quickly thawed at 37C^0 and washed thrice with 15 ml of RPMI-1640 washing medium at room temperature. The cells were then resuspended in 5 ml of RPMI-1640 supplemented with 1% GPPS and 10% FCS and transferred to a 25 cm^3 tissue culture flask and the cultures were grown at 37C^0 in a humidified incubator with 5% CO_2 in air mixture.

Phenol Equilibration:

Analytic grade Phenol was redistilled at 160C^0 to remove contaminants that cause breakdown or cross linking of nucleic acids and then stored in 500 ml aliquots at –20C^0. Before use phenol was equilibrated. First it was removed from the freezer and allowed to come at room temperature. Then the solid phenol was melted at 55C^0. 8-hydroxy quinoline was added as an antioxidant and RNAse inhibitor at a final concentration of 0.1% .The melted phenol was extracted once with equal volume 1.0 M Tris buffer pH 8.0, and then 3 to 4 times with 0.1 M Tris buffer pH 8.0, until the pH of the phenolic phase is greater than 7.8. Finally equal volume of 0.1 M Tris pH 8, containing 0.2% B-mercaptoethanol was added. The equilibrated phenol could be stored at 4C^0 (Sambrook, *et al.* 1989).

Preparation of Genomic DNA From Transformed Lymphocytes:

Genomic DNA was prepared using a modified organic extraction method (Maniatis *et al.* 1982). Cell pellets ($5x10^7$-10^8 cells) obtained from EBV transformation were gently vortex in a sterile 50 ml falcon tube. 19 ml of STE (saline tris EDTA) buffer was added, followed by 1ml of 10% SDS (drop wise while gently vortexing) and 20ul of (20g/ml) proteinase K. The samples were incubated over night at 55 C^o in a shaking water bath.

On the following day each sample was extracted with an equal volume (20ml) of equilibrated phenol, pH 8.0 by mixing gently on ice for 10 minutes, keeping on ice for 10 minutes. The samples were then centrifuged at 3200 rpm for 40 minutes at $4C^o$. The upper aqueous layer containing DNA was carefully collected in a 50ml falcon tube and extracted with an equal volume of chloroform-isoamyl alcohol (24:1 v/v) by cut-tips mixing gently on ice for ten minutes and keeping on ice for ten minutes. Then centrifuged at 3200 rpm for 40 minutes at $4C^0$. Again aqueous layer was collected in a fresh tube. The nucleic acids were precipitated by adding one-tenth (2ml) volume of 10M ammonium acetate followed by an equal volume (22ml) of chilled isopropanol. The samples were mixed until nucleic acids were visible (as floating white precipitate) and stored at $-20C^0$ over night or in $-70C^0$ for 15 minutes.

On the following day the precipitated nucleic acids were centrifuged at 3200 rpm for 90 minutes at $4C^0$ .The supernatant was decanted and the pellets were tapped with 5 ml of 70% ethanol. Then centrifuged at 3200 rpm for 40 minutes at $4C^0$. The supernatant was decanted and vacuum dried for 10 minutes. After drying resuspened in 1ml of TE buffer (Tris EDTA:10 mM Tris and 1mM EDTA; pH 8.0) and incubated at $37C^0$ in a shaking water bath for 1 hour. After 1 hour 10ul of RNAse A (10 mg/ml) was added to digest RNA and incubated at $37C^0$ for two hours in a shaking water bath. Then 50ul of 10 % SDS and 5ul of proteinase K was added and incubated the samples at $55C^0$ in a shaking water bath for one hour (after incubation samples may be stored over night at $4C^0$).

After incubation 4 ml of TE buffer was added and extracted with 5ml of equilibrated phenol, pH 8.0. The samples were mixed gently on ice for 10 minutes and kept on ice for 10 minutes. Then centrifuged at 3200 rpm for 40 minutes at $4C^0$. The aqueous layer was collected by cut tips in a fresh tube and 2 ml of TE was added to remove remaining phenol. The samples were mixed for 10 minutes on ice and kept on ice

for 10 minutes. Then centrifuged at 3200 rpm for 40 minutes at 4 C^0 .The aqueous layer was collected and extracted with 7 ml of chloroform-isoamyl alcohol mixed for 10 minutes and kept on ice for 10 minutes. Then centrifuged at 3200 rpm for 40 minutes at 4 C^0. The aqueous layer was removed and DNA was precipitated by adding one-tenth volume of 10 M ammonium acetate followed by two volumes of absolute ethanol or an equal volume of iso-propanol. The samples were mixed until DNA was visible. The sample was left over night at –20C° or at –70C° for 15 minutes.

The samples were centrifuged at 3200 rpm for 90 minutes at 4C°. The supernatant was removed and 5ml of 70% ethanol was added and pellets were tapped so it floated. Then the samples were spinned for 40 minutes at 3200 rpm and 4C°. The supernatant was decanted and the tube was inverted on tissue paper and vacuum dried for 10 minutes.1ml of 10mM Tris was added to resuspend the DNA and stored at 4C°.

Optical density of DNA samples was measured at 260nm. DNA concentration was calculated by the following formula:

DNA concentration ug/ml =Absorbance at 260nm x dilution factor x 50

(50= correction factor)

For pure quality DNA, the ratio between absorbance at 260nm and 280nm should be between1.7 to 2.0 (ratio greater than 2.0 indicate Phenol contamination and less than 1.7 indicate protein contamination) (Sambrook *et al.*1989). The genomic DNA was then transferred to a labeled eppendorf tube and stored at 4C°.

Preparation of Genomic DNA From Blood:

Genomic DNA was prepared using a modified organic extraction method (Maniatis *et al.* 1982). Freshly prepared chilled lysis buffer (containing 5mM MgCl2, 1% tritonX100; 10mMTris-HCl; 0. 32M Sucrose) was added to the blood samples in 3:1 ratio and centrifuged at 2500rpm 4C° for 15 minutes. The supernatant was discarded and 9.5ml of STE (saline tris EDTA) buffer (containing 100mM NaCl; 50mM Tris; 1mM EDTA; pH8.0) was added to the pellet, followed by the addition of 500ul of 10% SDS (drop wise while gently vortexing) and 10ul of proteinase K (20g/ml). The samples were incubated over night at 55 C° in a shaking water bath.

On the following day each sample was extracted with an equal volume (10 ml) of chilled equilibrated phenol (pH 8.0) by mixing gently on ice for 10 minutes, keeping on

ice for 10 minutes. The samples were then centrifuged at 3200 rpm for 30 minutes at 4C°. The upper aqueous layer containing nucleic acids were carefully collected in a fresh tube with cut tips without disturbing the interface layer of debris and extracted with an equal volume of chloroform-isoamyl alcohol (24:1 v/v) by cut-tips mixing gently on ice for ten minutes and keeping on ice for ten minutes. Then centrifuged at 3200 rpm for 30 minutes at $4C^0$. Again aqueous layer was collected in a fresh tube. The nucleic acids containing solution was then treated with 100ul of RNAse (10mg/ml) to digest RNA and incubated at 37 C° for two hours.

After incubation the samples were mixed with 500ul of 10 % SDS and 50ul of proteinase K and incubated the samples at $55C^0$ in a shaking water bath for one hour, after incubation samples were stored over night at $4C^0$. On the following day each sample was extracted with an equal volume (5 ml) of equilibrated phenol (pH 8.0) by mixing gently on ice for 10 minutes, keeping on ice for 10 minutes. The samples were then centrifuged at 3200 rpm for 30 minutes at 4C°. The upper aqueous layer containing nucleic acids were carefully collected in a fresh tube with cut tips and extracted with an equal volume of chilled chloroform-isoamyl alcohol (24:1 v/v) by cut-tips mixing gently on ice for ten minutes and keeping on ice for ten minutes. Then centrifuged at 3200 rpm for 30 minutes at $4C^0$.The aqueous layer was collected in a fresh tube by cut tips and 500 ul of 10M ammonium acetate and 5ml of chilled isopropanol was added to the samples and mixed until nucleic acids were visible (as floating white precipitate) and stored at $-20C^0$ over night or in $-70C^0$ for 15 minutes .On the following day The samples were centrifuged at 3200 rpm for 40 minutes at $4C^0$. The supernatant was removed and 5ml of 70% ethanol was added and pellets were tapped so it floated. Then the samples were centrifuged at 3200 rpm for 40 minutes at 4C°. The supernatant was decanted without disturbing the pellet and the tube was inverted on tissue paper and vacuum dried for 10 minutes. Then 10mM Tris was added to resuspend the DNA and stored at 4C°.

Optical density of DNA samples was measured at 260nm. DNA concentration was calculated by the following formula:

DNA concentration ug/ml =Absorbance at 260nm x dilution factor x 50

(50= correction factor)

For pure quality DNA, the ratio between absorbance at 260nm should be between 1.7 to 2.0 (ratio greater than 2.0 indicate Phenol contamination and less than 1.7 indicates protein contamination), Sambrook *et al.*1989. The genomic DNA was then transferred to a labeled eppendorf tube and stored at 4C°.

Micro satellite Analysis Using PCR:

For micro satellite analysis commercially available polymorphic markers were used (Research Genetics). Each reaction contained a total 10ul volume of PCR. For this purpose 8ul of master mix and 2ul of each DNA sample (40ng/ul) was prepared. Master mix was comprised of PCR buffer (sigma: containing 10mM TrisHCl, pH 8.3; 50mm KCl; 1.5mm $MgCl_2$; 0.01%(w/v) gelatin; 200uM dNTPs; 0.6uM each of forward and reverse primer and 0.2 U Taq polymerase per reaction. PCR was carried out using the following conditions:

1 cycle of	94C° for 4 minutes
35 cycles of	94C° for 45 seconds
	55C° for 45 seconds
	72C° for 45 seconds
1 cycle of	72C° for 10 minutes

The PCR products were kept at 4C° until electrophoresis was carried out.

Gel Electrophoresis:

The PCR products were separated on 10 % non-denaturing polyacrylamide gel. For gel electrophoresis Sequi-Gen GT Electrophoresis cell (Bio Red; Size 38x50cm) were used, the back plate (integral plate chamber, IPC) was siliconized by a siliconizing fluid (sigmacote) and the front plate was wiped with 70 % ethanol. The glass plates were assembled using 0.75mm spacers and held tightly using lever clamps.

Gel Casting:

10% Polyacrylamide gel was prepared by adding 62.5ml of 40% Bisacrylamide solution (containing 389.6grams of acrylamide and 10.4grams of N, N-methylene-bis-acrylamide dissolved per liter of the solution, stored at 4C° and 25 ml of 10 X TBE (Tris borate EDTA, 8.9mM Tris-borate; 2mM EDTA, pH8.0) and raised the volume up to 250ml with deionized water. Before gel casting base of the IPC assembly was blocked.

For this purpose the bottom edge of the assembly was fitted into a precision caster base. 50ml gel solution containing 300ul of 25% APS (Ammonium per sulphate) and 150ul of TEMED (N, N, N, N-tetra methyl ethylene diamine) was poured into the precision caster base and was left for 10 to 15 minutes to polymerize.

After blocking the base, the assembly was laid in a slanting position, and 200ml gel solution containing 850ul of 25% APS and 150ul of TEMED, was carefully poured in between the glass plates. A 68 well forming comb was inserted at the top of the assembly and the gel was allowed to polymerization for at least 2 hours or left over night.

After polymerization, precision caster base was removed and the IPC assembly was fitted in the universal base (Electrophoresis tank) using a stabilizer bar. The upper buffer chamber (IPC) and lower buffer chamber (universal base) were fitted with 1X TBE buffer and set at, pre run at 100 watts for 10 to 15 minutes. After pre-run the comb was gently removed and wells were washed thoroughly.

Loading the Gel:

Six ul of loading dye (containing 30% ficoll; 0.01% xylene cyanol and 0.001% bromophenol blue) was added to 10 ul DNA samples (PCR product). Eight ul of this mixture was loaded in each well. A DNA marker was also loaded.

Gel Running and Autoradiography:

The gel was run at 100W for 4 to 5 hours depending upon the size of PCR product. Gel assembly was then disconnected and carefully gel was separated and stained with ethidium bromide (5ul/100ml) for about 1 minute and photographed under UV illumination using syngene.

Allele Scoring:

The PCR products, visible on the photograph as bright DNA bands, were separated by electrophoresis on the basis of their sizes. Alleles were assigned to the individuals and were scored by giving them numbers according to their position on the photograph. Numbering started from the slowest running DNA band so that the fastest running DNA band was given the last number. Then the numbers given to the alleles of each individual were noted and haplotypes were constructed. The resultant haplotype data was used to calculate LOD score for each marker.

Linkage Analysis:

Linkage analysis is a powerful method of gene mapping. The primary goal is to determine if two or more genetic traits i.e. marker locus and a disease trait are co-segregating within a pedigree.

Linkage analysis was done using a software package LINKAGE version 5.2. Two-point linkage analysis was performed between the disease gene and each marker using M-link program. The disease was considered as Autosomal Recessive. The gene frequency was set at 1/100,000.The disease gene in the family was considered as full penetrant. The number of alleles for each marker was set as the number observed in the pedigree (Dracopoli *et al.*1995). Lod Score (Z) was calculated at recombination fraction 0.0,0.01,0.05,0.1, 0.2, 0.3, and 0.4.

RESULTS
CHAPTER-1

INTRODUCTION

LEBER'S CONGENITAL AMAUROSIS:

About 10 to 20% of children in institutions for the blind are thought to suffer from Leber's congenital amaurosis (LCA) (Alstrom, 1957; Phillips *et al.* 1987). The condition is estimated to account for 5% of inherited retinal disease (Mohamed *et al.* 2003). Currently, about 50% of patients are genetically accounted for by mutations in one of the known genes (Cremers *et al.* 2002)

CLINICAL DESCRIPTION:

Leber congenital amaurosis (LCA), first described by Theodore Leber in 1869, is the most severe form of early-onset inherited retinal dystrophy responsible for congenital blindness or severely impaired vision. Clinically, patients affected by LCA present in infancy with pendular nystagmus, unusual roving eye movements and absent ocular pursuit upon ophthalmic examination. LCA patients may habitually rub their eyes with the fist or fingers (the oculodigital reflex) (Franceshetti, 1947) and have a higher incidence of keratoconus than in the normal population. In early infancy, the optic discs and fundus are normal in appearance showing no abnormality upon fundoscopy. However, progressive abnormalities in the fundus appear with time, including attenuation of the retinal vasculature, optic nerve pallor, bone corpuscular pigmentation, atrophy of the retinal pigment epithelium (RPE) and occasionally irregular yellow pigmentation within the peripheral and mid-peripheral retina. In the absence of fundus abnormalities in early infancy, LCA is differentiated from other early-onset retinal dystrophies by electroretinographic (ERG) testing. The ERG recordings in LCA patients are markedly attenuated or absent. Additional systemic disorders, of which psychomotor retardation is the most frequent, have been reported in association with LCA (Moor and Taylor, 1984; Lambert *et al.* 1989).

Molecular genetics of Leber's congenital amaurosis (LCA):

LCA is a genetically heterogeneous disorder with an autosomal recessive mode of inheritance (Alstrom, 1957). Although infrequent accounts of dominant inheritance have been reported (Sorsby and Williams, 1960; Sohocki *et al.* 2000b; Rivolta *et al.* 2001; Perrault *et al.* 2003). To date, six causative genes have been identified in LCA (Table-8, pp. 43-44), the first

of which maps to chromosome 17p13.1 in humans (Camazut *et al.* 1995 and 1996) and translates to a photoreceptor-specific guanylate cyclase (retGC-1 or GUCY2D) (Perrault *et al.* 1996). RetGC-1 is an essential component of the phototransduction cascade, and mutations in retGC-1 impair the recovery of the dark state after photo-excitation of the photoreceptor cells. The situation is equivalent to sustained photo-excitation of the photoreceptors and patients with LCA caused by mutations in retGC-1 present with severe hyperopia and photophobia (Perrault *et al.* 1999).

The second locus for LCA was assigned to chromosome 1p31 (Hamel *et al.* 1994) and the affected gene encodes a microsomal membrane protein (RPE65) of the RPE (Marlhens *et al.* 1997). *RPE65* is a crucial component of the visual cycle in that it facilitates the isomerization of all-*trans*-retinylester to 11-*cis*-retinol and hence the regeneration of the universal chromatophore 11-*cis*-retinal (Hamel *et al.* 1993 and Tsilou *et al.* 1997). Mutations in the *RPE65* gene decrease the production of functional visual pigments leading to a situation equivalent to the sustained absence of photo-excitation of photoreceptor cells. Patients with LCA caused by mutations in RPE65 present with moderate or no hyperopia and some times low myopia upon ophthalmic examination (Perrault *et al.* 1999).

The third causative gene of LCA is a cone–rod homeobox (*CRX*) gene, which maps to chromosome 19q13.3 in humans (McInnes *et al.*1998. Freund *et al.*1997). The CRX protein is a photoreceptor-specific homeodomain transcription factor that plays an essential role in the differentiation, development and maintenance of photoreceptor cells through the transactivation of several photoreceptor-specific gene promoters, including rhodopsin (Freund *et al.* 1997, Furukawa *et al.* 1997, Chen *et al.* 1997).

The aryl hydrocarbon receptor interacting protein-like 1 (*AIPL1*) gene was the fourth gene found to cause LCA. *AIPL1* maps to within 2.5 Mb distal to the retGC-1 locus on chromosome 17p13.1 in humans (Sohocki *et al.* 2000b). The *AIPL1* gene encompasses six exons encoding a protein of 384 amino acids in length. The protein sequence includes three consecutive 34 amino acid tetratricopeptide repeat (TPR) motifs, which are thought to mediate specific protein interactions. TPR motifs are ubiquitously conserved in structurally unrelated proteins that participate in diverse biological functions, including the co-ordination of multiprotein complex assembly and protein translocation (Blatch and lassle, 1999). A 56 amino acid polyproline-rich sequence of high flexibility encompassing multiple *O*-glycosylation sites

and putative phosphorylation sites is present at the C-terminus of the AIPL1 protein in humans. The function of AIPL1 in normal vision is unknown. However, AIPL1 is similar (49% identity) to the aryl hydrocarbon receptor-interacting protein (AIP) (Ma and Whitlock 1997), also known as the aryl hydrocarbon receptor-activated protein (ARA9) (Carver and Bradfield, 1997) or the X-associated protein (XAP2) (Kuzhandaivelu *et al.* 1996). The aryl hydrocarbon receptor is a cytosolic ligand-activated transcription factor that mediates adaptive and toxic responses to environmental pollutants such as dioxin by increasing the transcription of xenobiotic metabolizing enzymes. AIP facilitates the transactivation activity of the cognate transcription factor by regulating its nuclear translocation The inclusion of the TPR motifs in the AIPL1 protein and the similarity of AIPL1 to AIP collectively suggest that AIPL1 may be involved in retinal protein folding or cellular translocation.

Recently, two other LCA causative genes have been identified, *CRB1* (1q31.3) (Lotery *et al.* 2001,den Hollander *et al.*2001) and *RPGRIP1* (14q11.2) (Dryja *et al.* 2001). CRB1 is a retina and CNS expressed protein that is homologous to the *Drosophila* crumbs protein that is important in cell polarity and cell–cell contact, although the role of CRB1 in the retina remains to be identified. RPGRIP1 was identified as an interacting partner of the X-linked retinitis pigmentosa protein RPGR and is thought to be localized to the photoreceptor connecting cilium where it may play a role in transport between the inner and outer segments. The diverse nature of these LCA causative genes illustrates the complexity of retinal cell biology and highlights that many, as yet unstudied, pathways are essential for the normal function of the retina.

Recently a new locus for LCA (LCA9) has been found in a single Pakistani family which maps to chromosome 1p36 (Keen *et al.* 2003).

LCA is not a curable disorder. Research, at molecular level is in progress. Genes responsible for this disorder have been reported from different populations. Pakistani population is an interesting one because of close relative marriage and particularly in first cousin marriages, which are preferred here. Because of inbreeding in human population it is expected that recessive form of disorder shall appear. The aim of the present study is to search for any new locus for this disorder and how consanguinity influences in the appearance of this disorder. These contributions in this regard may be helpful in future research regarding gene therapy of this disorder.

RESULTS:

Epidemiology:

A large Pakistani kindred suffering from congenital blindness was identified (Fig.5). The surname of the family is Abbasi and they are settled in District Rawalpindi. The members of the family were not well educated. The kindred were very cooperative in providing relevant information they were asked. Their marriages were contracted both among cousins and distant relatives. The kindred spans on five generations and consists of seventy seven individuals, out of which sixty two were live and fifteen were dead at the time of data collection. Of these live members, thirteen members were affected including six females and seven males.

Individual V-3 is the proband, which was first diagnosed for the disease and with his help the disorder was traced back in the family.

In the first generation, a marriage between distantly related normal individuals, I-1 and I-2 produced four normal sons (II-3, II-5, II-6 and II-9). Another marriage was between un-related individuals I-3 and I-4 who had two normal daughters (II-7 and II-8).

In the second generation all marriages were contracted between normal individuals. Marriage between II-1 and II-2 produced one normal daughter (III-2) and two normal sons (III-3 and III-6). Second marriage was between II-3 and II-4 who had two normal daughters (III-4 and III-19) and two normal sons (III-8 and III-10). The third marriage was between II-6 and II-7 they had two normal sons (III-12 and III-13) and two normal daughters (III-14 and III-15). Fourth marriage in this generation was between II-8 and II-9 who had two normal sons (III-16 and III-17) and one normal daughter (III-18).

In the third generation all marriages were contracted between normal un-related individuals with the exception of two first cousins marriages and one first cousin once removed marriage. Marriage between III-1 and III-2 produced one normal daughter (IV-1). Marriage between III-3 and III-4 produced one normal son (IV-2). Individual III-6 was married twice, once with III-5 and had one affected daughter (IV-3), one normal daughter (IV-4), one affected son (IV-5) and one normal son (IV-6); from his second marriage with III-7, he had one normal daughter (IV-7) and one normal son (IV-8). Marriage between III-10 and III-11 produced two normal daughters (IV-10 and IV-12) and one normal son (IV-11). A woman III-19 married to normal man (III-20) had one normal daughter (IV-19) and one normal son (IV-20). Consanguineous marriage between first cousins III-8 and III-9 was noticed in the third generation. They were both normal

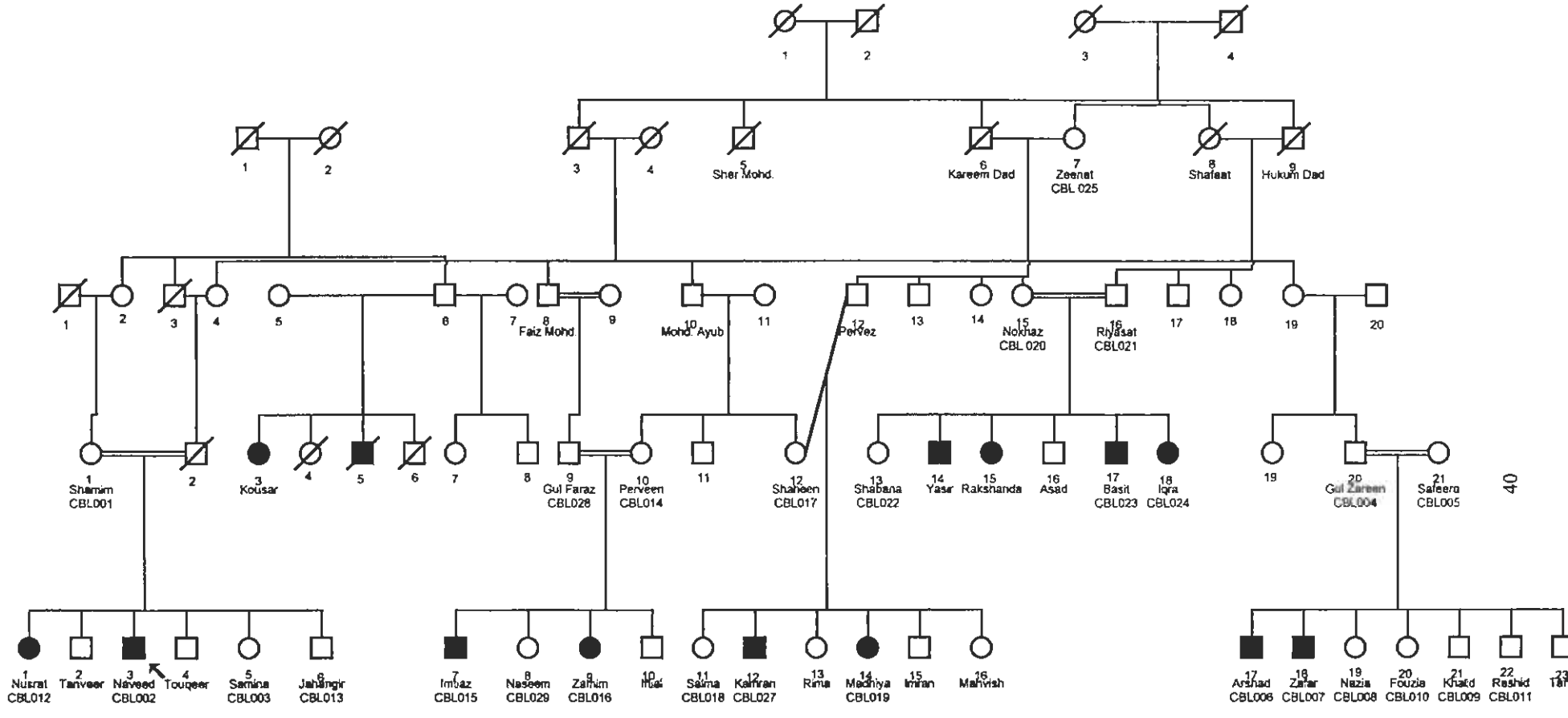


Fig.5. Pedigree of a consanguineous Pakistani family suffering from autosomal recessive Leber's congenital amaurosis (LCA), 3CBL. Segregation proportion of the disease is approximately 3:1

and had one normal son (IV-9). Another consanguineous marriage between normal first cousins III-15 and III-16 had one normal daughter (IV-13), two affected daughters (IV-15 and IV-18), two affected sons (IV-14 and IV-17) and one normal son (IV-16). There was one first cousin once removed marriage between normal man III-12 and normal woman IV-12, they had three normal daughters (V-11, V-13 and V-16), one normal son (V-15), one affected son (V-12) and one affected daughter (V-14).

In the fourth generation there were three first cousin marriages. First marriage was between normal individuals IV-1 and IV-2, they produced one affected daughter (V-1), three normal sons (V-2, V-4 and V-6), one normal daughter (V-5) and one affected son (V-3). Second first cousin marriage was between normal individuals IV-9 and IV-10, which resulted in one affected son (V-7), one affected daughter (V-9), one normal daughter (V-8) and one normal son (V-10). Third consanguineous marriage was between IV-20 and IV-21 who were both normal and had two affected sons (V-17 and V-18), two normal daughters (V-19 and V-20) and three normal sons (V-21, V-22 and V-23).

The pedigree shows an autosomal recessive mode of inheritance for this disorder.

Clinical assessment:

Two affected members (IV-15 and V-9) of the family 3CBL were clinically examined. Their ages were 15 years and 10 years respectively.

Age of onset:

All the affected members of the family had congenital blindness.

Clinical description:

The ophthalmologist noted following signs of the disorder in affected individuals.

1. Pupillary light reflexes were absent and corneal opacities at peripheral part were present.
2. Fundus showed diffused patches of peripheral chorioretinal atrophy and granulity.
3. A 'salt-and-pepper' fundus.
4. Maculopathy was observed in both patients.
5. Optic disc pallor and attenuation of the retinal arterioles.
6. Nystagmus, roving eye movements and strabismis.
7. Cortical cataract stage I and II appeared in both affected individuals.
8. ERG was non-recordable.

Parent observations:

They reported that Individuals with ocular disorder were congenitally blind.

Associated abnormalities:

Affected individuals IV-14, IV-17 and V-14 were mentally retarted, epileptic, and with severe neurological disorders. In other affected individuals and normal individuals no other abnormality like polydactly, obesity, hypogonadism, hearing loss and other neurological disorders were found.

Proposed diagnosis:

The proposed diagnosis made by the clinician is Leber congenital amaurosis.

Molecular Studies:

Epidemiology of the family 3CBL has already been described (Fig.5, pp.40). Peripheral blood samples were collected from 29 individuals for molecular studies. These include 11 affected and 18 normal individuals. Those marriages, which were informative regarding segregation for blindness trait were included for analysis at molecular level. Of eleven blind individuals six were males and five females. Blood samples were numbered as shown in the pedigree (Fig.5).

Initially the affected members and their first-degree relatives were screened to exclude the linkage to the regions of the previously described genes involved in LCA on chromosomes 1,6, 14, 17 and 19 (Table 8; pp. 43-44).

All the known loci were excluded except LCA4 locus (17p13.1). Co-segregation of the disease region with markers D17S938 and D17S796 was found. In order to confirm the linkage, flanking markers of this region were typed for the whole family. DNA samples amplified with these markers for affected and normal individuals were loaded in the polyacrylamide gel in the following order.

CBL001, CBL012, CBL002, CBL 003, CBL013, CBL028, CBL014, CBL015, CBL016, CBL029, CBL017, CBL018, CBL019, CBL027, CBL020, CBL021, CBL022, CBL023, CBL024, CBL026, CBL004, CBL005, CBL006, CBL007, CBL008, CBL009, CBL010 and CBL011.

Table-8. Known loci, genes and frequency of Leber's Congenital Amaurosis (LCA) as reported by other authors.

Disease	Gene	locus	Available markers	Frequency	References
Recessive Leber congenital amaurosis; Recessive RP	CRB1	1q31.3	D1S413, D1S1660	9-13%	denHollander *et al.*2001; Lotery *et al.*2001;Heckenlively *et al.* 1982; Leutelt *et al.* 1995; van Soest *et al.* 1994.
Recessive Leber congenital amaurosis; Recessive RP	RPE65	1p31.2	D1S201(ED)	7-16%	Acland *et al.* 2001;. Gu *et al.* 1997 ; Marlhen *et al.* 1997; Morimura *et al.* 1998 ; Van Hooser *et al.* 2000.
Recessive Leber's congenital amaurosis	Unknown	1p36	D1S1612 D1S228	One consanguineous Pakistani family.	Keen *et al.* 2003.
Recessive Leber congenital amaurosis	Unknown	6q11-q16	D6S460 (F) D6S462 (F)	Limited number of consanguineous families	Dharmaraj *et al.* 2000; Mohamed *et al.*2003
Recessive Leber congenital amaurosis	RPGRIP1	14q11.2	D14S261 D14S1023 D14S1043 D14S742	6%	Dryja *et al.* 2001;Cremers *et al* 2002;Hameed *et al.*2003.
Recessive	Unknown	14q24	D14S606(8)	Homozygosity	Stockton *et al.* 1998

Leber congenital amaurosis			GATA30Ho4(8)	mapping : consanguineous Saudi Arabian family	
Recessive Leber congenital amaurosis; dominant cone-rod dystrophy	GUCY2D	17p13.1	D17S796(ED) D17S1353(ED)	6%	Camuzat *et al.* 1995; Camuzat *et al.* 1996; Perrault *et al.*1996; kelsell *et al.* 1997; Lotery *et al.*2000; Perrault *et al.* 1998.
Recessive Leber congenital amaurosis; dominant cone-rod dystrophy	AILP1	17p13.1	D17S796(ED) D17S938(ED)	10%	Hameed *et al.* 2000;Sohocki *et al* 2000b.
dominant cone-rod dystrophy; Recessive, dominant and denovoLeber congenital amaurosis, dominant RP,	CRX	19q13.3	D19S220(F) D19S420(F)	3%	Freund *et al.*1998; Jacobson *et al.*1998; Swaroop *et al.*1999; Rivolta *et al.*2001; Perrault *et al.* 2003.

It was ascertained from gel photograph (Fig.6) that two markers (D17S1298 and D17S1303) did not amplify for one individual (CBL013), whereas, with other markers amplification was noted for all individuals.

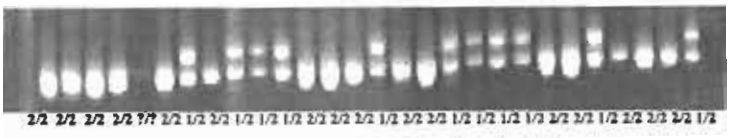
The microsatellite markers D17S1298, D17S938, D17S796, and D17S1303 were typed for confirmation of linkage at LCA4 locus.

HAPLOTYPE ANALYSIS:

Nineteen microsatellite markers were used. Of these seventeen markers were excluded and linkage was shown at LCA4 locus with two markers (D17S938; D17S796).

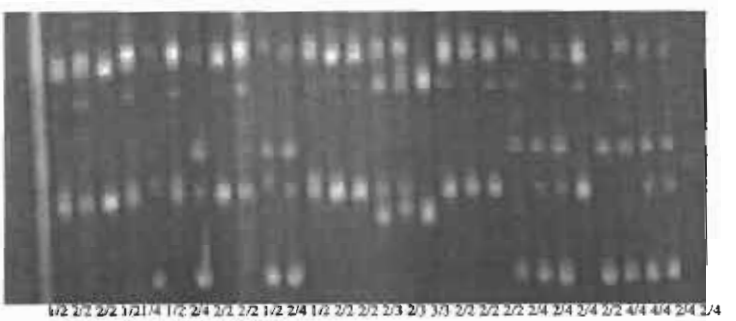
Alleles were assigned to the individuals and their haplotypes are shown in the Fig.7. Two point LOD score analysis resulted in maximum LOD score (Z max) of 4.75 for marker D17S796 at recombination fraction 0 (Table 9). The flanking marker D17S938 proximal to D17S796 and spaced at 0cM gave LOD score 2.32 at recombination fraction 0. Another marker D17S1298 proximal to D17S796 and spaced at 3.87 cM yielded LOD score of 0.29 at recombination fraction 0.2. Marker D17S1303 distal to marker D17S796 and spaced at 8.87 cM resulted in a negative LOD score of $-\infty$ at recombination fraction 0 and it was -3.52 at recombination fraction 0.01.The highest negative lod score for this marker was -0.03 at recombination fraction 0.4. Two point LOD score and genetic distances between markers are listed in Table-9.

All affected individuals are homozygous for marker D17S796 (IV-7, IV-8, IV-9, V-1, V-2, V-5, V-7, V-9, V-10, and V12) except one individual (V-11). The affected individuals hetrozygous for marker D17S1298 and D17S1303 are IV-7, IV-8, IV-9, V-7, and V-12. The affected individuals hertozygous for marker D17S1303 are V-2, V-5, V-9 and V-10. There is only one affected individual (V-1) who is homozygous for all the four markers (D17S1298, D17S938, D17S796, and D17S1303). In all blind individuals the haplotype region between markers D17S938 and D17S796 is homozygous, except V-11 who is hetrozygous for marker D17S796. Other markers while segregating (D17S1298 and D17S1303) appear both homozygous and heterozygous. In affected members IV-7, IV-8; IV-9, V-7 and V-12 recombination at markers between D17S1298 and D17S938, and between D17S796 and D17S1303 has been observed. Affected members V-2, V-5, V-9, V-10, and V-11, show recombination between markers D17S796 and D17S 1303.

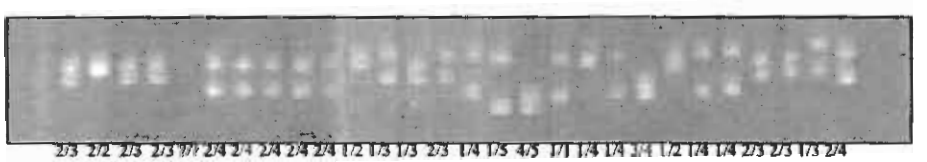


D17S1298

D17S938



D17S796



D17S1303

Fig.6. Gel photograph of the markers, D17S1298, D17S938, D17S796 and D17S1303 used for LCA4 in the family 3CBL.

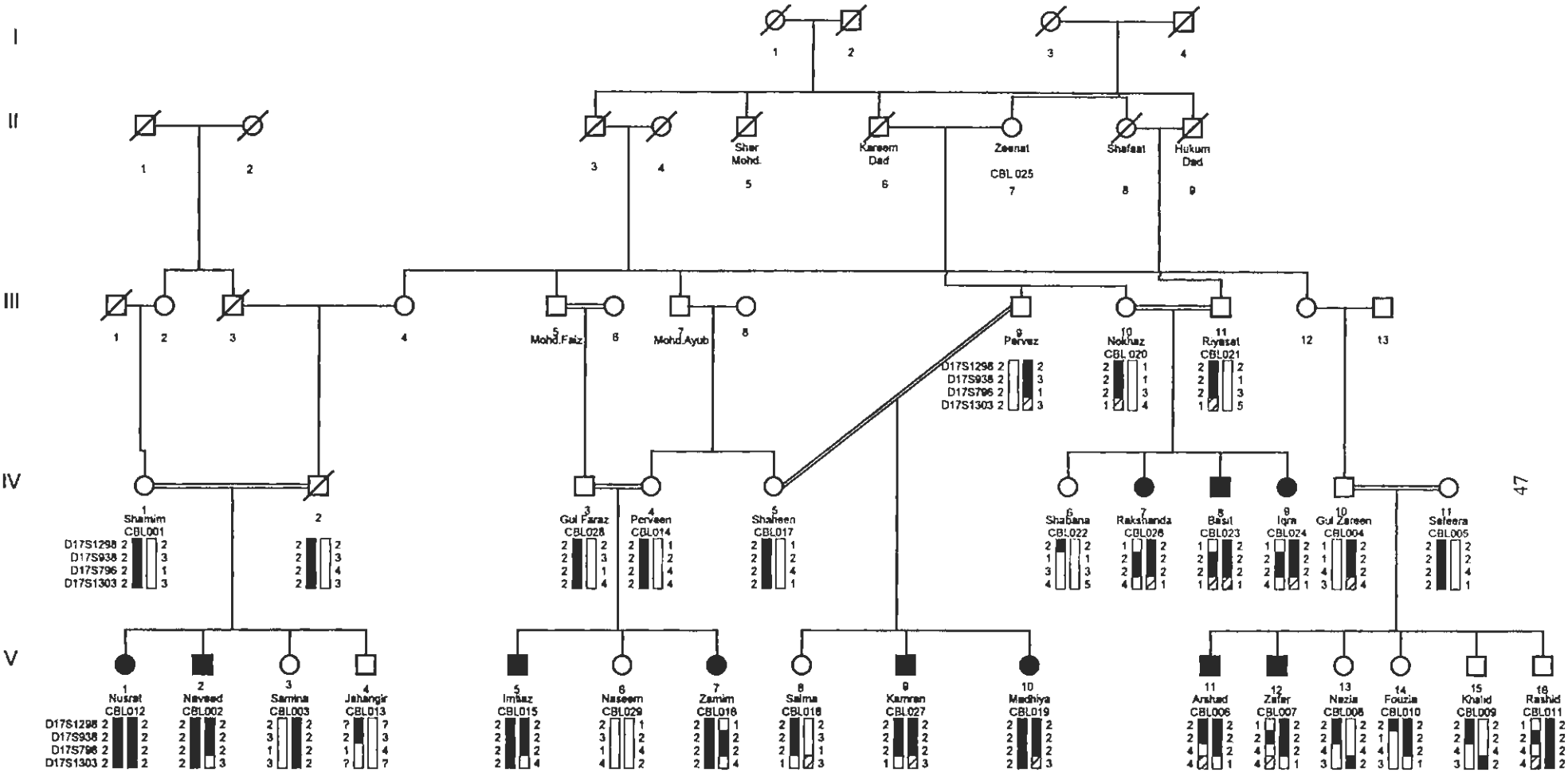


Fig.7. Part of pedigree 3CBL(Fig.5) showing homozygosity of the disease haplotype in affected individuals. Shaded bars denote the disease haplotype, and cross line represent the cross overs.Haplotypes of III-12 and IV-2 are assumed from their progeny.

Table-9: Two point lod scores and the genetic distances between the microsatellite markers analysed in this family.

Marker	Marshfield map position (cM)	LOD scores at θ								
		0.0	0.01	0.05	0.1	0.2	0.3	0.4	Zmax	θ max
D17S1298	10.72	-∞	-0.69	-0.02	0.21	0.29	0.19	0.06	0.29	0.2
D17S938	14.69	2.32	2.27	2.07	1.79	1.21	0.62	0.15	2.32	0.0
D17S796	14.69	4.75	4.63	4.13	3.48	2.18	1.04	0.25	4.75	0.0
D17S1303	23.56	-∞	-3.52	-1.52	-0.78	-0.25	-0.08	-0.03	0.03	0.4

Occurrence of recombination at markers D17S1298 and D17S938; and between D17S796 and D17S1303 isolate the critical region of marker D17S796 that in all affected members remains homozygous (except V-17).

RESULTS:

4CBL

Epidemiology:

A Pakistani kindred suffering from congenital blindness was identified (fig. 8). The surname of the family is Raja and they are settled in District Rawalpindi. The members of the family were not well educated. The kindred were very cooperative in providing relevant information they were asked. Their marriages were contracted both among cousins and distant relatives. The kindred spans on five generations and consists of twenty nine individuals out of which twelve were live and seventeen were dead at the time of data collection. Of these live members, five members were affected including two females and three males.

Individual V-4 is the proband, which was first diagnosed for the disease and with his help the disorder was traced back in the family.

In the first generation, a marriage between distantly related normal individual, I-1 and I-2 produced one normal daughter (II-2) and one affected son (II-3).

In the second generation a first cousin marriage between normal individuals (II-1 and II-2) produced one normal son (III-1) and one normal daughter (III-2). Marriage between an affected individual (II-3) and normal individual (II-4) produced five normal sons (III-3, III-4, III-6, III-7 and III-9) and two normal daughters (III-8 and III-10).

In the third generation a first cousin marriage was contracted between normal individuals (III-2 and III-3) and they produced one normal daughter (IV-1) and two normal sons (IV-2 and IV-3). Another marriage between normal unrelated individuals (III-4 and III-5) produced two normal daughters (IV-4 and IV-6) and one normal son (IV-5). In the fourth generation there was a first cousin marriage between (IV-3) and (IV-4), they produced two affected daughters (V-1 and V-5), two normal sons (V-2, and V-3) and three affected sons (V-4, V-6 and V-7). The pedigree shows an autosomal recessive

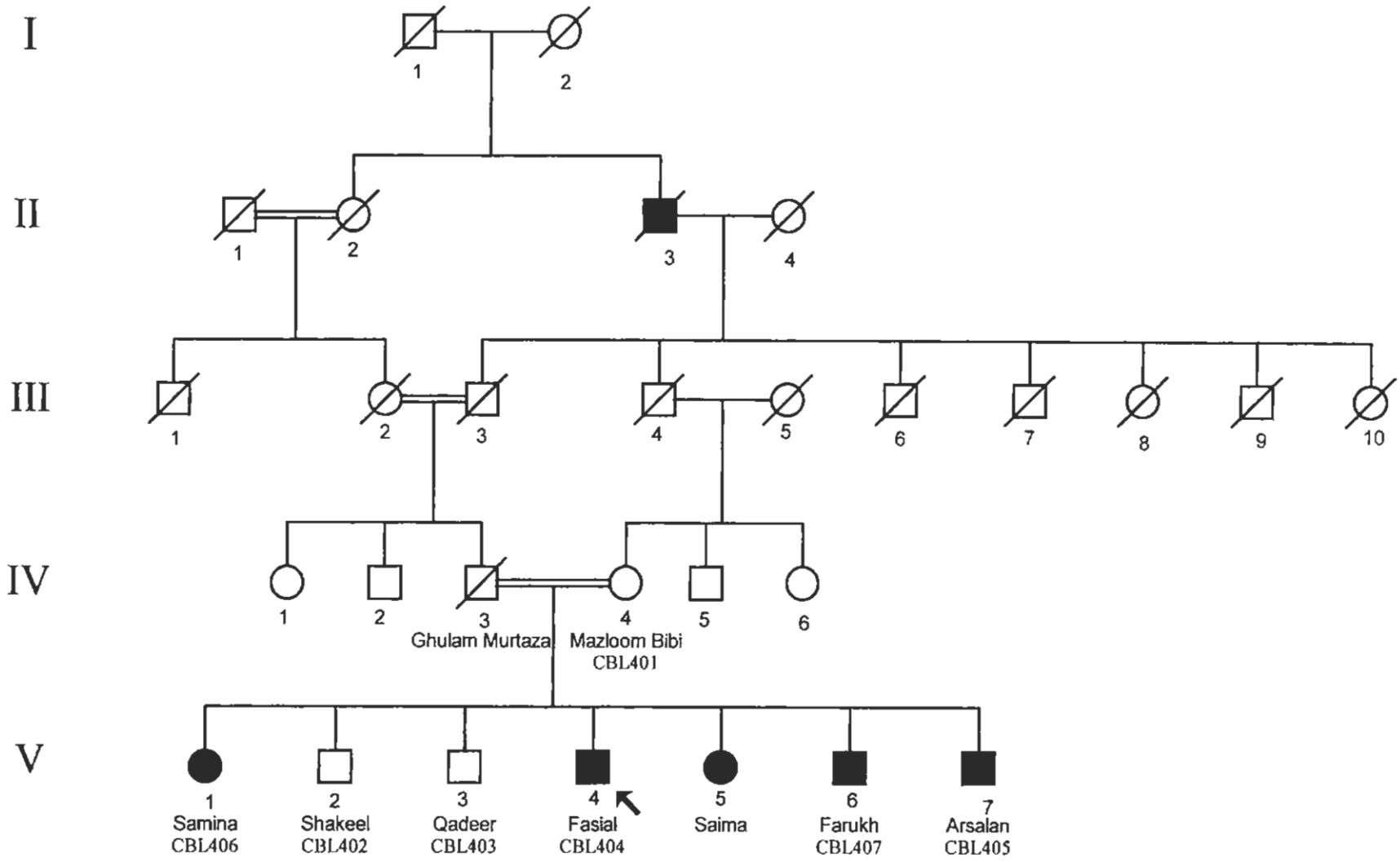


Fig.8 Pedigree of a consanguineous Pakistani family suffering from autosomal recessive Leber's congenital amaurosis (LCA),4CBL.

mode of inheritance for this disorder.

Clinical assessment:

Three affected members (V-4 and V-6 and V-7) of the family 4CBL were clinically examined. Their ages were 22 years and 18 and 15 years respectively.

Age of onset:

All the affected members of the family had congenital blindness.

Clinical description:

The ophthalmologist noted following signs of the disorder in affected individuals.

1. Pupillary light reflexes were absented in V-4 and V-6, whereas poor perception of light was present in V-7.

1. Fundus showed disc oedema, macular coloboma and maculopathy.
2. Optic disc pallor and attenuation of the retinal arterioles in all affected individuals.
3. Nystagmus, roving eye movements and strabismis was observed in all affected individuals.
4. ERG was non-recordable.
5. Individual V-4 had one eye microphthalmos and other maldeveloped.

Parent observations:

They reported that individuals with ocular disorder were congenitally blind.

Associated abnormalities:

Affected individual V-5 and normal individual V-3 were mentally retarted, physically handicapped, with severe skeletal abnormalities and dwarf, whereas all other affected individuals were of short stature and with bowing of legs and wide wrist. Other associated abnormalities like polydactly, obesity, hypogonadism, hearing loss, etc. was not found.

Proposed diagnosis:

The proposed diagnosis made by the clinician is Leber's congenital amaurosis (LCA).

Molecular Studies:

Epidemiology of the family 4CBL has already been described (Fig.8; pp.50). Peripheral blood samples were collected from seven individuals for molecular studies. These include four affected and three normal individuals. Of four blind individuals three

were males and one female. Blood samples were numbered as shown in the pedigree (Fig.8).

Initially the affected members and their first-degree relatives were screened to exclude the linkage to the regions of the previously described genes involved in LCA disorder on chromosomes 1,6, 14, 17 and 19 (Table-8; pp.43-44).

All the known loci were excluded except LCA4 locus (17p13.1). Co-segregation of the disease region with marker D17S1832 was found. In order to confirm the linkage, flanking markers of this region were typed for the whole family. DNA samples amplified with these markers for affected and normal individuals were loaded in the polyacrylamide gel in the following order.

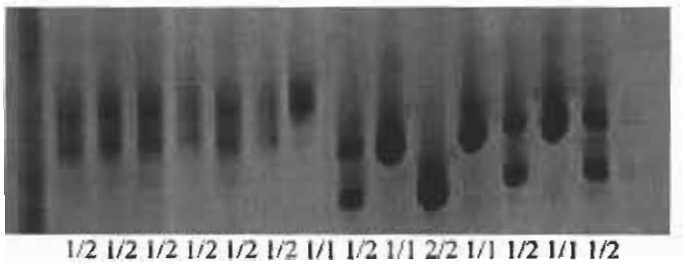
CBL401, CBL406, CBL403, CBL 402, CBL407, CBL404, CBL405.

The microsatellite markers D17S1308, D17S1298, D17S1832, D17S796, D17S974 and D17S1303 were used for confirmation of linkage at LCA4 locus (Fig.9).

HAPLOTYPE ANALYSIS:

Co-segregation of the disease region with marker D17S1832 was found. Alleles were assigned to the individuals and their haplotypes are shown in the Fig.10. All markers were informative except marker D17S796.

Two point LOD score analysis resulted in maximum LOD score (Z max) of 2.06 for marker D17S1832 at recombination fraction, $\theta = 0$ (Table 10). The flanking marker D17S1298 is placed proximal to D17S1832 and is spaced at 2.35cM gave homozygosity with two affected individuals (CBL406 and CBL404) and hetrozygosity with another two affected individuals (CBL407 and CBL405) whereas a normal individual CBL402 also showed homozygosity with this marker. Therefore, it is difficult to interpret that this marker showed a linkage with the disease region. Another marker D17S1308 proximal to D17S1832 and spaced at 12.44 cM showed homozygosity with only one affected individual CBL405 whereas other affected individuals CBL406, CBL404, CBL407 showed heterozygous genotype. Marker D17S974 distal to marker D17S1832 and spaced at 9.17 cM resulted in a heterozygous genotype in all affected individuals thus showing no linkage with the disease region. Another marker D17S1303 also distal to D17S1832 and spaced at 10.49cM also showed lack of linkage with the disease region.



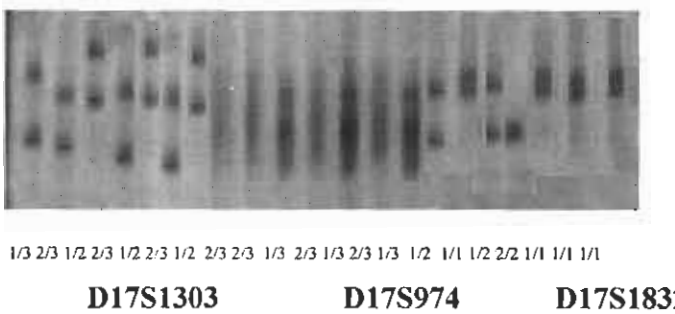


Fig.9: Gel photograph of markers, D17S1308, D17S1298, D17S1303, D17S974 and D17S1832 used in the family, 4CBL.

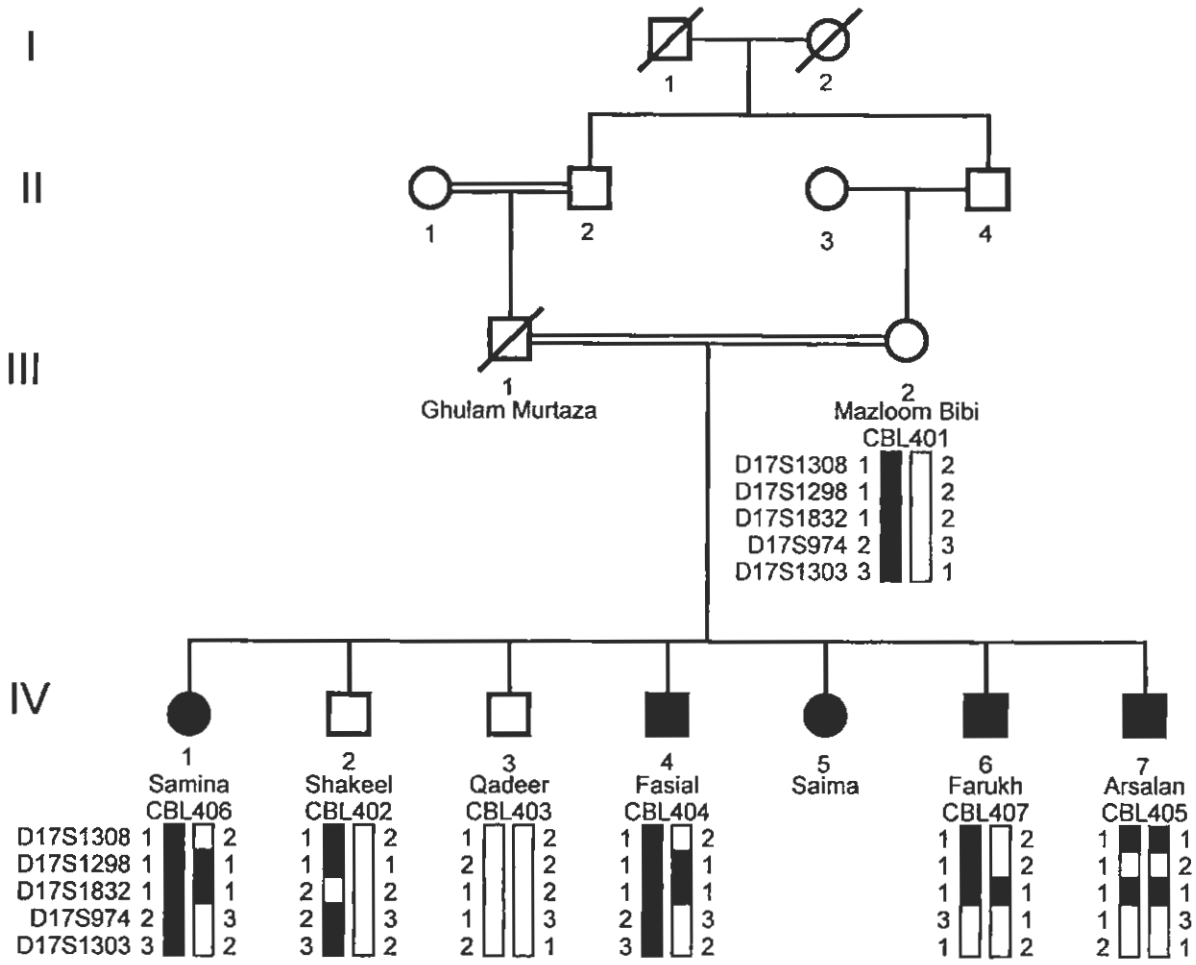


Fig.10 Part of pedigree 4CBL (Fig.8), showing homozygosity of the disease haplotype in affected individuals.Shaded bars denote the disease haplotype.

Table-10. Two point lod scores and the genetic distance of the microsatellite markers analysed in the family (4CBL).

Marker	Marshfield map position (cM)	LOD scores at θ								
		0.0	0.01	0.05	0.1	0.2	0.3	0.4	Zmax	θ max
D17S1308	0.63	-∞	-1.16	-0.51	-0.26	-0.07	-0.01	-0.00	-0.00	0.4
D17S1298	10.72	-∞	-2.26	-0.94	-0.43	-0.05	0.04	0.02	0.04	0.3
D17S1832	13.07	2.06	2.02	1.86	1.64	1.19	0.70	0.23	2.06	0.0
D17S974	22.24	-∞	-∞	-∞	-∞	-∞	-∞	-∞	-∞	0.0
D17S1303	23.56	-∞	-∞	-∞	-∞	-∞	-∞	-∞	-∞	0.0

The highest lod score for marker D17S1832 was 2.06 at recombination fraction $\theta = 0.00$, which was homozygous for all affected individuals. Linkage analysis isolates the critical region of marker D17S1832 which in all affected members remains homozygous. Marker D17S1832 seems to be linked with the disease region, but lod score is less than 3 due to less number of informative meioses and also unfortunately the tightly linked marker (D17S796) of the LCA4 locus was non- informative for this family.

DISCUSSION:

Leber congenital amaurosis is the designation for a group of autosomal recessive retinal dystrophies that represent the most common genetic causes of congenital visual impairment in infants and children. LCA is characterized by moderate to severe visual impairment identified at or within a few months of birth, infantile nystagmus, sluggish pupillary responses (and occasionally a paradoxical pupil response), and absent or poorly recordable electroretinographic responses early in life. Additional features include symmetric midfacial hypoplasia with enophthalmos and hypermetropic refractive errors. There is substantial variation between families and intrafamilial similarities.

Present study revealed the presence of disease locus at LCA4 region (17P13.1). Two point linkage analysis showed the maximum lod score (Z max) of 4.75 with microsatellite marker D17S796 at $\theta = 0$ in the family 3CBL and the maximum lod score (Z max) of 2.06 with microsatellite marker D17S1832 at $\theta = 0$ in the family 4CBL.

At this locus two candidate genes (AIPL1 and RetGC-1) are present. Due to the proximity of AIPL1 and RetGC-1 linkage mapping may not distinguish between the genes. Further, it is possible that LCA patients who are identicle by descent (IBD) at one locus are also IBD at the other. Therefore, it is suggested that both AIPL1 and RetGC-1 should be screened for mutations in families whose LCA locus maps to 17p13.

Sohocki *et al.* (2000a) reported the presence of LCA locus on 17p13.1. In one Pakistani family they mapped the LCA locus to 17p13.1, between D17S849 and D17S960, a region that excludes GUCY2D. The LCA in this family was designated as LCA4. They described a new photoreceptor/pineal-expressed gene, AIPL1 (encoding aryl-hydrocarbon interacting protein-like 1), that maps within the LCA4 candidate region

and whose protein contains three tetratricopeptide (TPR) motifs, consistent with nuclear transport or chaperone activity. They described that a homozygous nonsense mutation at codon 278 is present in all affected members of the LCA4 family. They described that AIPL1 mutations may cause approximately 20% of recessive LCA. This is in agreement with the present study as in present study maximum lod score (Z max) of 4.75 was obtained with microsatellite marker D17S796 at $\theta = 0.0$ in the family 3CBL, and a maximum lod score of 2.06 with microsatellite marker D17S1832 at $\theta = 0.0$ in the family 4CBL, an area that excludes RetGC, involvement of AIPL1 is possible.

Sohocki *et al.* (2000b) analyzed that microsatellite markers D17S796 and D17S1881 are tightly linked flanking markers of AIPL1 gene. In contrast, microsatellite markers D17S960 and D17S1353 are flanking markers of RetGC-1 gene. As in present study maximum lod score (Z max) of 4.75 was obtained with microsatellite marker D17S796 at $\theta = 0$ in the family 3CBL, showing the exclusion of the region of RetGC-1 and possible involvement of AIPL1 gene. Similarly family 4CBL showed a maximum lod score of 2.06 with microsatellite marker D17S1832 at $\theta = 0$, also exclude the region of RetGC-1 gene, therefore involvement of AIPL1 is also possible in the family 4CBL.

Balciuniene *et al.* (1995) demonstrated linkage between progressive cone dystrophy, designated CORD5, and and genetic markers on chromosome 17p13-p12. Multipoint analysis gave a maximum lod score of 7.72 at the marker D17S938. A recombination was detected between the gene encoding the retinal protein recoverin, thus excluding it as the site of the mutation. Two other candidate genes, located at 17p13.1, are retinal guanylate cyclase and pigment epithelium-derived factor. Moreover, loci for Leber congenital amaurosis and for a form of retinitis pigmentosa map to the same region. Present study also indicate the linkage of microsatellite markers D17S796 with a maximum lod score of 4.75 at $\theta = 0$ and D17S938 with a lod score of 2.32 at $\theta = 0$ in the family 3CBL and a maximum lod score of 2.06 with microsatellite marker D17S1832 at $\theta = 0$ in the family 4CBL, revealed the presence of disease locus at 17p13.1.

Camuzat *et al.* (1995) mapped a gene for Leber congenital amaurosis to the distal short arm of chromosome 17 by linkage analysis in 15 multiplex families with maximum

lod score = 5.14 at $\theta = 0.15$ for a probe at the D17S1353 region. When they split the collection of families into 2 groups according to the ethnic origin of the patients, they were able to confirm the presence of a gene for LCA on 17p by both homozygosity mapping and linkage analysis in 5 families of Maghrebian origin; (maximum lod score = 7.21 at $\theta = 0.01$ at the D17S1353 region, while negative results were found in 10 families of French ancestry. Haplotype analyses supported the placement of the gene to which they designated LCA1, between D17S796 and D17S786. From the location of the markers, they concluded that the LCA1 gene in the North African families is located at 17p13. The linkage demonstration of genetic heterogeneity in LCA confirmed the conclusion of Waardenburg and Schappert-Kimmijser (1963) based on the observation of normal children born to 2 affected parents. Camuzat *et al.* (1995) stated that the genes on distal 17p that are good candidate genes for LCA1 include recoverin, beta-arrestin 2 ,retinal guanylate cyclase (GUC2D), and phosphatidylinositol transfer protein. Camuzat *et al.* (1996) further refined the LCA1 locus to a 1-cM region on chromosome 17p between markers D17S938 and D17S1353.

Perrault *et al.* (1996) demonstrated mutations in the GUC2D gene in French families as the cause of type I Leber congenital amaurosis in some but not all families. They identified 2 missense mutations and 2 frameshift mutations in GUC2D. Sohocki *et al.* (2000a) identified mutations in the AIPL1 gene, which, like GUCY2D, maps to 17p13, as the cause of type IV Leber congenital amaurosis.

Of families with GUCY2D mutations as the basis of Leber congenital amaurosis, 70% originate from Mediterranean countries, the remaining families originating from various countries around the world. Hanein *et al.* (2002) identified a homozygous 2943G deletion in the GUCY2D gene in 3 unrelated and non-consanguineous Leber congenital amaurosis families of Finnish origin, suggesting a founder effect. No linkage disequilibrium was found using polymorphic markers flanking the GUCY2D gene, supporting the view that the mutation is very ancient. Haplotype studies and Bayesian calculation pointed the founder mutation to 150 generations (i.e., 3,000 years ago).

Ehara *et al.* (1997) reported a previously undescribed autosomal recessive syndrome in 4 Japanese children from 2 unrelated families. All 4 children had Leber congenital amaurosis, short stature, developmental delay, hepatic dysfunction, and metabolic acidosis. Three of the 4 children had decreased growth hormone secretion. The karyotype was normal in all 3 children in which it was tested. One of the children died without growth hormone secretion or karyotype having been assessed. Two of the 4 patients were monozygotic twins. One patient underwent a muscle biopsy at age 11 years to look for evidence of a mitochondrial disorder. Mild variation in fiber size and type 2 fiber atrophy were seen on histopathologic examination, but no ragged-red fibers were observed. No mitochondrial DNA alterations were found. Autosomal recessive inheritance was suggested on the basis of 2 sibs born to healthy parents. In neither family were the parents consanguineous. Present study revealed the presence of autosomal recessive inheritance on the basis of 5 affected individuals born to normal parents. The parents were first cousins. In the family 4CBL all affected individuals had short stature, one normal for LCA and one blind individual had severe mental retardation, and all affected individuals had bowing legs.

Yano *et al.* (1998) reported 2 sisters, born of first-cousin parents, with Leber congenital amaurosis, cerebellar vermis hypoplasia, and facial dysmorphism including hypertelorism, short philtrum, thin upper lip, and prominent jaw. Both were severely mentally retarded with abnormal behavior. Mild skeletal abnormalities consisted of limited extension of elbows and fingers and talipes equinovalgus. One sister had a scalp skin defect and renal anomalies. The authors postulated that their report may represent a distinct clinical entity or a severe manifestation of one of the described LCAs. Present study is also in agreement with the above findings as most of the symptoms were present in one brother and one sister of the family 4CBL born of first-cousin parents.

Lotery *et al.* (1996) identified a large Northern Irish pedigree in which 19 individuals in 3 generations had central areolar choroidal dystrophy (CACD). Through a total genome search for linked markers, they established linkage of CACD in this family to chromosome 17p (maximum lod = 4.7 at $\theta = 0$ with D17S796). In a study of the same family, Hughes *et al.* (1998) refined the critical region to an interval of approximately 5 cM flanked by polymorphic markers D17S1810 and CHLC GATA7B03. Hughes *et al.*

(1998) noted that the ARRB2 gene maps to 17p13 but had not yet been finely localized. Present study also showed a linkage at 17p13.1 an area of AIPL1 gene with markers D17S796 and D17S938 in the family 3CBL and with marker D17S1832 in the family 4CBL.

Several mutations in the LCA4 and adCORD cases have been reported in different exons of the AIPL1 gene (Sohocki *et al.*2000 a,b; Damji *et al.* 2001).They also suggested that of these Trp278x mutation appears to be very common and has been reported in several families from different ethnic backgrounds around the world, including six families of Pakistani origin. The presence of a novel Thr39 Asn mutation in this gene in yet another Pakistani family suggests a high rate of AIPL1 gene mutations in Pakistani families (Khaliq *et al.* 2003). Many LCA families of Pakistani origin mapped on $17p^{13}$ have been shown to have AIPL1 gene mutations. The interesting observation is that none of the $17p^{13.1}$ linked Pakistani families that had been reported so far, had disease associated mutations in the GUCY2D gene (Khaliq *et al.* 2003). It is suggested that AIPL1 is the candidate gene, which is involved in the pathogenesis of autosomal recessive leber congenital amaurosis.

Based on reports regarding gene AIPL1 in different populations of the world including Pakistani families, it is suggested that AIPL1 is the candidate gene for Leber congenital amaurosis, which has been diagnosed, in autosomal recessive form in both the families. The family 4CBL was diagnosed with additional forms like short stature, skeletal abnormalities bowing of legs and one affected individual (V-4) with one micropthalmic eye and other maldeveloped eye.

RESULTS
CHAPTER-2

INTRODUCTION

Macular degeneration:

Macular degeneration can be divided into two broad categories:

1: Age-related macular degeneration.

2: Early onset macular degeneration.

Age-related macular degeneration:

Age-related maculopathy (ARM) is a common disorder of the macular retina accounting for the majority of blindness and partial sight in the developed world. Age-related macular degeneration is the major cause of severe visual impairment in the population of over 60 years of age and is increasing for unknown reasons. It also have a significant genetic component to its etiology. Putative susceptibility loci have been identified on chromosome 1q25-q31(Klein *et al.*, 1998; Weeks *et al.*, 2001), chromosome 17q25 (Weeks *et al.*, 2001) and on chromosomes 5,9 and 10 (Weeks *et al.*, 2000). It has been suggested that the e4 allele of the apolipoprotein E gene and an Alu polymorphism in the angiotensin-converting enzyme gene may have a protective effect on ARMD risk (Klaver *et al.*, 1998; Hamdi *et al.*, 2002).

Early onset macular degeneration:

Early onset forms are inherited macular degenerations and are looked upon as macular dystrophies instead of degeneration. Macular dystrophy is a condition, which appears earlier in life, and causes a reduction in the central vision found in macula, the central portion of retina. Macular dystrophies are usually inherited in a predictable pattern within families. There is genetically determined abnormality causing either the loss of the normal pigment associated with the photoreceptors in the retina or the accumulation of an abnormal protein (Prevost, 2000). The inherited macular dystrophies are characterised by bilateral visual loss and the finding of generally symmetrical macular abnormalities on ophthalmoscopy. The age of onset is variable, but most present in the first two decades of life. There is considerable clinical and genetic heterogeneity: macular dystrophies showing autosomal dominant, autosomal recessive, X-linked recessive and mitochondrial inheritance have all been reported (Michaelides *et al.* 2003a).

AUTOSOMAL RECESSIVE INHERITANCE

Stargardt disease and fundus flavimaculatus:

Stargardt macular dystrophy (STGD) is the most common inherited macular dystrophy with a prevalence of 1 in 10,000 has autosomal recessive mode of inheritance. It shows a very variable phenotype because of variable age of onset and severity. Most cases present with central visual loss in early teens and there is typically macular atrophy with white flecks at the level of the retinal pigment epithelium (RPE) at the posterior pole on ophthalmoscopy. The prognosis is relatively poor. Once the patient's visual acuity drops below 6/12, it tends to decrease rapidly and then stabilizes at about 6/60 to 3/60. Stargard disease may also present in adult life when the visual loss may be milder (Kanski, 1999).

The locus for STGD/FFM (Stargardt disease and fundus flavimaculatus) was mapped to chromosome 1p using homogeneity mapping in inbred families, and the causative gene characterized was ATP-binding cassette transporter-retinal (ABCR) (Fishman *et al.,* 1987). ABCR encodes a transmembrane rim protein located in the disc of rod and foveal cone outer segments, that is involved in ATP dependent transport of retinoids from photoreceptor to RPE (Sun and Nathans,1997; Molday *et al.* 2000; Weng *et al.* 1999). Failure of this transport results in deposition of a major lipofuscin fluorophore, A2E (N-retinylidene-N-retinylethanolamine), in the RPE (Weng *et al.*, 1999). It is proposed that this accumulation may be deleterious to the RPE, with consequent secondary photoreceptor degeneration. Subsequently, mutations in ABCR have been implicated in other disorders, including retinitis pigmentosa (Martinez *et al.*, 1997; Martinez *et al.*, 1998) and cone-rod dystrophy (CORD) (Cremers *et al.*, 1998; Maugeri *et al.,* 2000).

AUTOSOMAL DOMINANT INHERITANCE

Autosomal dominant Stargardt-like macular dystrophy:

The clinical apperance of autosomal dominant Stargardt-like macular dystrophy is so similar to the common autosomal recessive form of the disorder that it is difficult to differentiate between them by fundus examination alone (Donoso *et al.*, 2001). However individuals reported with features of ad STGD like dystrophy have a milder phenotype with relative good functional visio, minimal colour vision defects and no significant electro-oculography (EOG) or elcctro-retinogram (ERG) abnormalities (Donoso *et al.*, 2001).

Two chromosomal loci have been identified, 6q14 (STGD3) and 4p (STGD4) (Stone *et al.*1994 and Kniazeva *et al.* 1999). Two mutations, a 5-bp deletion and two 1-bp deletions separated by four nucleotides, in the gene ELOVL4 (protein involved in elongation of very long fatty acids) have been associated with STGD3 and other macular dystrophy phenotypes including pattern dystrophy (Zhang *et al.* 2001; Bernstein *et al.* 2001). ELOVL4 is expressed in the rod and cone photoreceptor inner segments. The protein product is believed to be involved in retinal fatty acid metabolism since it has significant homology to a family of proteins involved in fatty acid elongation.

Michaelides *et al.* (2003b) have recently reported a British family with an autosomal dominant "Bull's-Eye" macular dystrophy (MCDR2) also mapping to chromosome 4p and overlapping the STGD4 disease interval reported by Kniazeva *et al.* 1999.

Best disease (vitelliform macular dystrophy):

Best disease is autosomal dominantly inherited macular dystrophy, which is characterized clinically by the classical features of a round or oval yellow subretinal macular deposit. The disease shows very variable expressivity. Most individuals carrying mutations in the VMD2 gene on chromosome 11q13 (Forsman *et al.*1992; Petrukhin *et al.*1998; Caldwell *et al.*1999) have an abnormal electro-oculography (EOG), but the macular appearance may be normal in some (Mohler and Fine, 1981). The variable expression of best disease remains unexplained, other genes in addition to VMD2, and or environmental influences may play a role in the wide range of clinical expression seen.

Adult vitelliform macular dystrophy:

Adult vitelliform macular dystrophy (AVMD) presents bilateral, round or oval, yellow, symmetrical, sub-retinal lesions, typically one third to one half optic discs diameter in size. Mutations in the peripherin RDS gene on chromosome 6p have been identified in AVMD (Felbor *et al.* 1997). It has been proposed mutations in peripherin RDS are present in approximately 20% of patients with AVMD (Felbor *et al.* 1997) which implies further genetic heterogeneity.

Pattern dystrophy:

The pattern dystrophy are a group of inherited disorders of the RPE which are characterized by bilateral symmetrical yellow-orange deposits at the macula in various

distributions, including butterfly or reticular-like patterns. These dystrophies are often associated with a relatively good visual prognosis, although in some cases a slowly progressive loss of central vision can occur. Mutations in the peripherin/RDS gene on chromosome 6p have been identified in patients with pattern dystrophies (Nichols *et al.*1993; Weleber *et al.*1993).

Doney honeycomb retinal dystrophy (malattia leventinese; autosomal dominant drusen):

In this disorder small round yellow-white deposits under the retinal pigment epithelium (RPE) are characteristically distributed at the macula and around the optic disc, and begin to appear in early adult life. Visually acuity is maintained through the fifth decade, but the patients usually become legally blind by the seventh decade. Visual loss is usually due to macular atrophy, but less commonly may follow subretinal neovascular membrane (SRNVM). The presence of drusen- like deposits makes this dystrophy potentially very relevant to age related macular degeneration (ARMD). A single mutation, Arg345Trp (R345W) in the gene EFEMP1 on chromosome 2p has been identified in the majority of patients with dominant drusen (Stone *et al.* 1999).

Autosomal dominant drusen and macular degeneration (DD):

Affected infants and children may have congenital atrophic maculopathy and drusen. The gene for the disease has been mapped to chromosome 6q14 and appears to be adjacent to but distinct from the locus for North Carolina macular dystrophy (MCDR1) (Kniazeva *et al.* 2000)

North Carolina macular dystrophy:

North Carolina macular dystrophy (MCDR1) is an autosomal dominant disorder, which is characterised by a variable macular phenotype and a non-progressive natural history. Linkage studies have mapped MCDR1 to a locus on chromosome 6q16. An early onset autosomal dominant macular dystrophy (MCDR3) resembling MCDR1 has been recently mapped to chromosome 5p (Michaelides *et al.*2003c)

Progressive bifocal chorioretinal atrophy (PBCRA):

PBCRA is an autosomal dominant disorder characterized by nystagmus, myopia and progressive macular and nasal retinal atrophic lesions. PBCRA has been linked to 6q14-q16.2 (Kelsell *et al.* 1995).

Sorsby fundus dystrophy (SFD) is a rare, autosomal dominant macular dystrophy, with onset of night blindness in the third decade and loss of central vision from macular dystrophy or SRNVM by the fifth decade. The tissue inhibitor of metalloproteinase-3 (TIMP3) gene on chromosome 22q is implicated in SFD (Weber *et al.*1994; Felbor *et al.* 1995; Jacobson *et al.* 2002)

Central areolar choroidal dystrophy (CACD):

CACD is characterised by bilateral, symmetrical, subtle mottling of the retinal pigment epithelium (RPE) at the macula in the early stages. The mottling then progresses to atrophy of the RPE and choriocapillaris. An Arg142Trp mutation in peripherin/RDS has been implicated as one cause of this rare autosomal dominant macular dystrophy (Hoyng *et al.* 1996). Sporadic cases of CACD have also been described but no mutations were found in peripherin/RDS (Hoyng *et al.* 1996). A second locus at chromosome 17p13 has also been identified by a genome wide linkage search in a large Northern Irish family (Lotery *et al.*1996; Hughes *et al.* 1998).

Dominant cystoid macular dystrophy (dominant cystoid macular oedema):

This rare disorder includes onset usually in the fourth decade, typically a moderate to high hypermetropic refractive error, and normal ERG. Genetic linkage has been established to 7p15-p21(Kremer *et al.* 1994). The causative gene remains to be identified.

In addition to those described above there are several other autosomal dominant macular dystrophies whose phenotypes are not well described.

X-LINKED INHERITANCE

X-linked juvenile retinoschisis (XLRS):

XLRS is a vitreoretinal degeneration, which presents either in an infant with nystagmus, or commonly in childhood with mild loss of central vision. The characteristic fundus abnormality is a cystic spokewheel-like maculopathy in virtually all affected males. Peripheral retinal abnormalities include bilateral schisis cavities, vascular closure, inner retinal sheen, and pigmentary retinopathy are seen in approximately 50% of cases. XLRS has been linked to Xp22.2 and mutations in the gene XLRS1 have been identified (Sauer *et al.* 1997).

MITOCHONDRIAL INHERITANCE

Maternally inherited diabetes and deafness (MIDD):

Macular pattern dystrophy (MPD) has been found in association with MIDD (Goto *et al.*1990). In a multicentre study, 86% of MIDD patients was found to have bilateral MPD, characterised by RPE hyperpigmentation that can surround the macula or be more extensive and also encompass the optic disc. As the prevalence of MPD in MIDD is high, the association of a MPD should raise the possibility of screening for mutation of mitochondrial DNA.

Macular degeneration is the leading cause of blindness in the world. Research, at molecular level is in progress. Genes responsible for this disorder have been reported from different populations. Pakistani population is an interesting one because of close relative marriages and particularly in first cousin marriages, which are preferred here. Because of inbreeding in human population it is expected that recessive form of disorder shall appear. The aim of the present study is to search for any new locus for this disorder and how consanguinity influences in the appearance of this disorder. These contributions in this regard may be helpful in future research regarding gene therapy of this disorder.

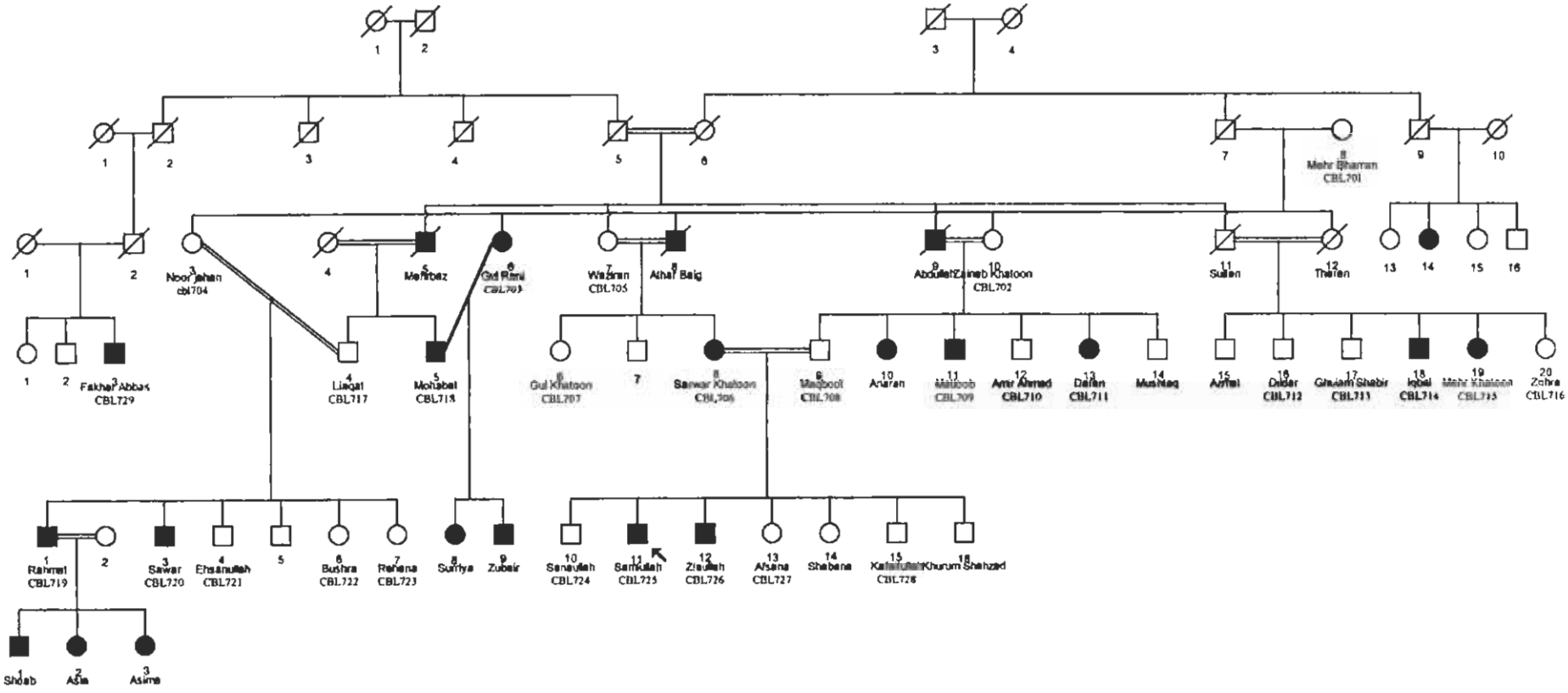
RESULTS:

Epidemiology:

A large Pakistani kindred suffering from congenital blindness was diagnosed (Fig.11). The surname of the family is Mirasi and they are settled in the District Mianwali. The members of the family are very poor, un-educated and were very cooperative in providing relevant information they were asked. Mostly consanguineous marriages were contracted within the kindred resulting in a higher number of affected individuals. The kindred spans on six generations and consists of 69 individuals, of which 48 were alive and 21 were dead at the time of data collection. Of these 48 live individuals 22 were affected, including 13 males and nine females.

Individual V-11 is the proband, who was first diagnosed for the ocular disorder (congenital blindness) and with his help the disorder was traced back in the family.

In the first generation there were two un-related marriages between normal individuals. First marriage was between (I-1) and (I-2) and had four normal sons (II-2, II-



ig.11. Pedigree of a consanguineous Pakistani family suffering from autosomal recessive macular degeneration, 7CBL.

3, II-4 and II-5). Another marriage was between (I-3) and (I-4) and produced one normal daughter (II-6) and two normal sons (II-7 and II-9).

In the second generation there was one first cousin marriage and three un-related marriages between normal couples. First cousin marriage was between (II-5) and (II-6) who produced two affected sons (III-5 and III-9), one normal daughter (III-7) and one normal son (III-11). One un-related marriage was between (II-1) and (II-2) and produced a normal son (III-2). Another un-related marriage was between (II-7) and (II-8) and produced three normal daughters (III-3, III-10 and III-12), one affected daughter (III-6) and one affected son (III-8). Third un-related marriage was between (II-9) and (II-10) and had two normal daughters (III-13 and III-15), one affected daughter (III-14) and one normal son (III-16).

In the third generation there was one un-related marriage, two one first cousin once removed marriages and four first cousin marriages. Un-related marriage was between normal couple (III-1 and III-2) and produced a normal daughter (IV-1), a normal son (IV-2) and an affected son (IV-3). One first cousin once removed marriage was between (III-3) and (IV-4), both were normal for blindness and produced two affected sons (V-1 and V-3), two normal sons (V-4 and V-5) and two normal daughters (V-6 and V-7). Another first cousin once removed marriage was between affected woman (III-6) and affected man (IV-5) who produced an affected daughter (V-8) and an affected son (V-9). One first cousin marriage was between normal woman (III-4) and affected man (III-5) who produced one normal son (IV-4) and an affected son (IV-5). Second first cousin marriage was between normal woman (III-7) and affected man (III-8) who produced one normal daughter (IV-6), one normal son (IV-7) and an affected daughter (IV-8). Third first cousin marriage was between affected man (III-9) and normal woman (III-10) who produced three normal sons (IV-9, IV-12 and IV-14), two affected daughters (IV-10 and IV-13) and one affected son (IV-11). fourth first cousin marriage was between normal couple (III-11 and III-12) who produced three normal sons (IV-15, IV-16 and IV-17), one affected son (IV-18), one affected daughter (IV-19) and one normal daughter (IV-20).

In the fourth generation first cousin marriage between affected woman (IV-8) and normal man (IV-9) produced three normal sons (V-10, V-15, and V-16), two normal daughters (V-13 and V-14) and two affected sons (V-11 and V-12).

In the fifth generation first cousin marriage between affected man (V-1) and normal woman (V-2) produced one affected son (VI-1) and two affected daughters (VI-2 and VI-3).

This pedigree shows an autosomal recessive mode of inheritance.

Clinical assessment:

Two affected members (V-8 and V-9) of the family 7CBL were clinically examined. Their ages were 17 years and 14 years respectively.

Age of onset:

All the affected members of the family had congenital blindness.

Clinical description:

Clinical description of the affected individuals is summarized in the Table-11.

Table-11. Clinical description of the affected individuals in the family 7CBL.

Patient No.	Visual Acuity		Bilateral nystagmus	Poor pupillary reflex	Macular degeneration	Attenuated blood vessels	Hypermetropic astigmatic optic disc
	Right	Left					
V-8	3/60	2/60	+	+	+	+	+
V-9	2/60	3/60	+	+	+	+	+

Ophthalmologist observed bilateral nystagmus, poor pupillary reflexes and visual acuity of 3/60, 2/60 and 2/60, 3/60 in right and left eye of V-8 and V-9, respectively. No improvement was seen with glasses. Retina on fundus examination of both affected individuals showed hypermetropic astigmatic optic disc and attenuated blood vessels. In V-8 maculae were degenerated and less pigmented with no rim of chorioretinal atrophy. Whereas, in V-9 central part of maculae was degenerated and more pigmented with a rim of chorioretinal atrophy.

Parents observations:

Parents reported that individuals with ocular disorder had reduced vision since birth.

Associated abnormalities:

Any associated abnormality likes polydactly, obesity, hypogonadism, hearing loss, mental retardation, epilepsy, neurological disorders etc. was not found among them. They were normal physically and mentally except ocular disorder.

Proposed diagnosis:

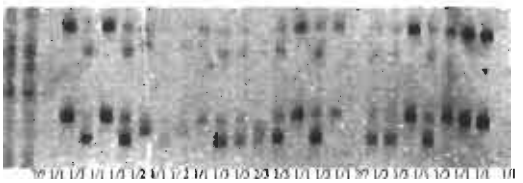
The proposed diagnosis made by the clinician is macular degeneration.

Molecular Studies:

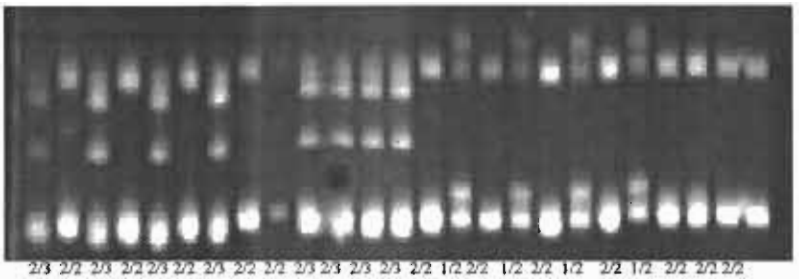
The members of this family were diagnosed for macular degeneration. Peripheral blood samples were collected from twenty-eight individuals with informed consent of the family for molecular studies. Blood samples were numbered as shown in the pedigree (Fig.11). Since the affected individuals were congenitally blind, therefore, initially the affected members and their close degree relatives were screened to exclude the linkage to the regions of the previously described genes involved in LCA on chromosomes 1,6,14,17 and 19 (Table-8; pp.43-44). During exclusion analysis, all the known loci for LCA were excluded. After exclusion analysis of known loci for retinal degeneration were searched and a linkage was observed at $6p^{21.2}$ region. More markers in this region were analyzed to obtain proximal and distal crossovers to localize the disease region. DNA samples amplified with the markers (D6S276, D6S291, D6S1610, D6S1019, and D6S1017) were loaded in the polyacrylamide gel in the following order:

CBL702, CBL709, CBL710, CBL711, CBL708, CBL706, CBL724, CBL725, CBL726, CBL727, CBL728, CBL705, CBL707, CBL703, CBL704, CBL717, CBL723, CBL720, CBL721, CBL718, CBL713, CBL714, CBL715, CBL719.

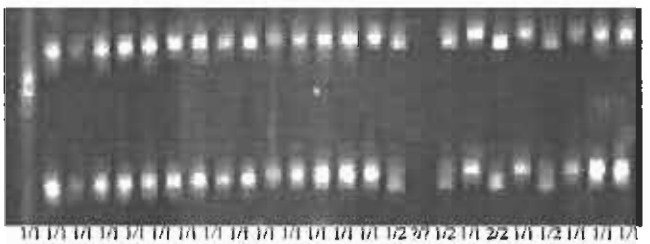
Fig.12 represent the gel photographs of markers analyzed in this region. Alleles were assigned to the individuals and their haplotypes are shown in the Fig.13. The details of markers in this region (D6S276, D6S291, D6S1610, D6S1019 and D6S1017) are given in the table-12. Two point lod score gives maximum value of 3.29 for marker D6S1019 at recombination fraction 0. Other higher values of lod score are 3.24 at recombination fraction 0.01 and 3.01 at recombination fraction 0.05 for marker D6S1019. The flanking marker D6S1610 proximal to marker D6S1019 is spaced at 0 cM which gave 2.44 lod score at recombination fraction 0. The flanking marker D6S1017 distal to marker D6S1019 is spaced at 9.47 gave maximum lod score of 3.00 at recombination fraction 0.05. Other high lod scores of 2.14 at recombination fraction 0.01 and lod score



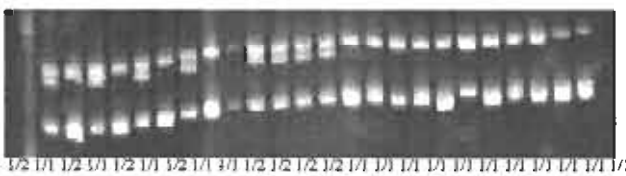
D6S276



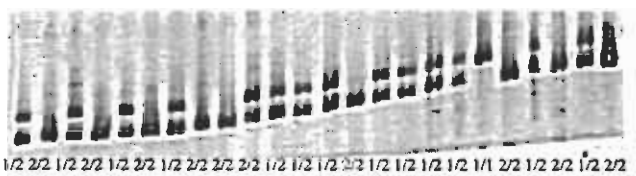
D6S291



D6S1610



D6S1019



D6S1017

Fig.12. Gel photograph of the markers, D6S276, D6S291, D6S1610, D6S1019 and D6S1017 used for macular degeneration in the family 7CBL.

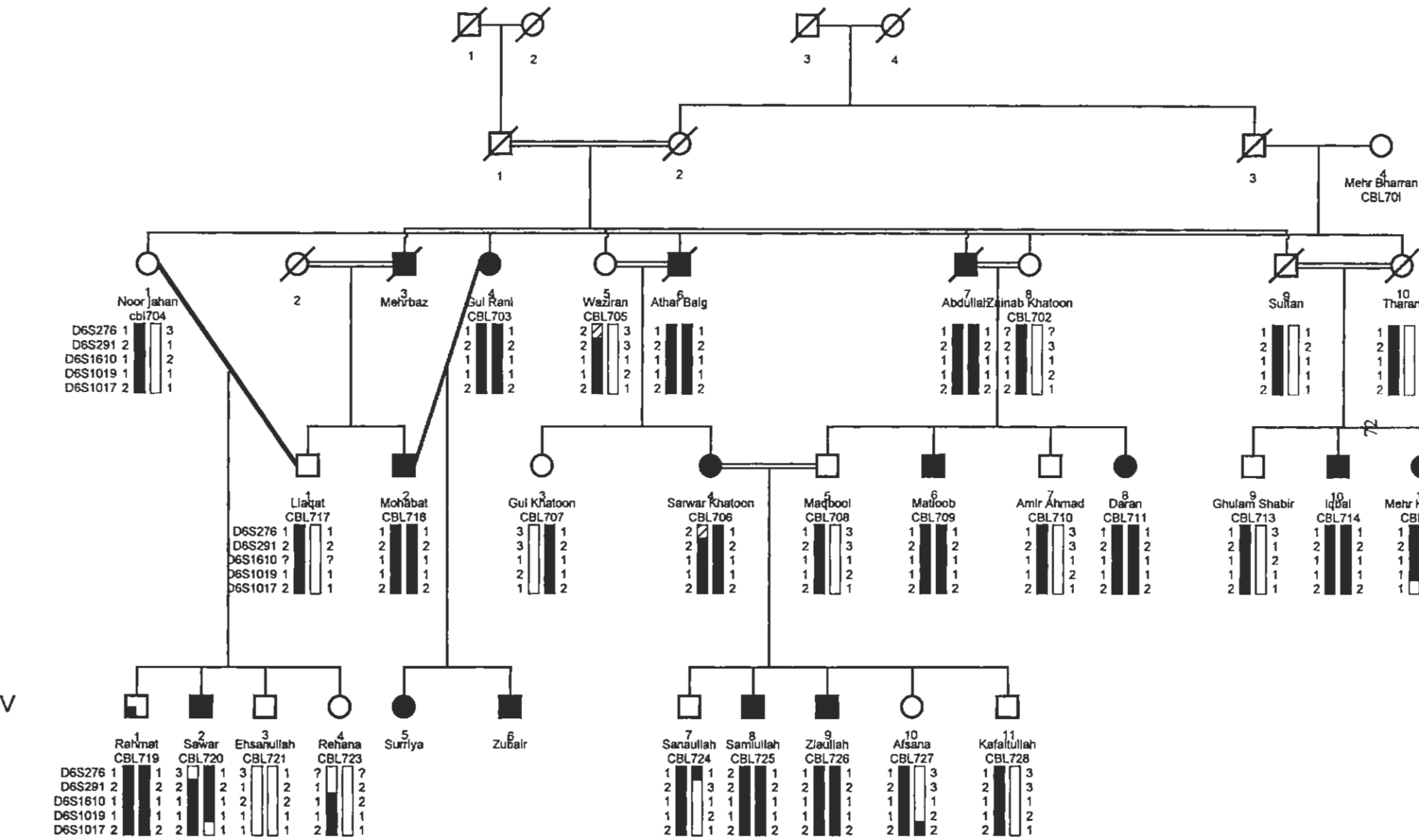

1
2
3
4
1
2
3
4
Mehr Bharran
CBL701
1
Noor Jahan
cbl704
D6S276 1 3
D6S291 2 1
D6S1610 1 2
D6S1019 1 1
D6S1017 2 1
2
3
Mehrbaz
4
Gul Rani
CBL703
5
Waziran
CBL705
6
Athar Baig
7
Abdullah
8
Zainab Khatoon
CBL702
9
Sultan
10
Tharan
1
Liaqat
CBL717
D6S276 1 1
D6S291 2 2
D6S1610 ? ?
D6S1019 1 1
D6S1017 2 1
2
Mohabat
CBL718
3
Gul Khatoon
CBL707
4
Sarwar Khatoon
CBL706
5
Maqbool
CBL708
6
Matloob
CBL709
7
Amir Ahmad
CBL710
8
Daran
CBL711
9
Ghulam Shabir
CBL713
10
Iqbal
CBL714
V
1
Rahmat
CBL719
D6S276 1 1
D6S291 2 2
D6S1610 1 1
D6S1019 1 1
D6S1017 2 2
2
Sawar
CBL720
3
Ehsanullah
CBL721
4
Rehana
CBL723
5
Sumiya
6
Zubair
7
Sanaullah
CBL724
8
Samiullah
CBL725
9
Ziaullah
CBL726
10
Afsana
CBL727
11
Kafaitullah
CBL728

Table-12 Two point lod scores and the genetic distances between the microsatellite markers analysed in the family 7CBL.

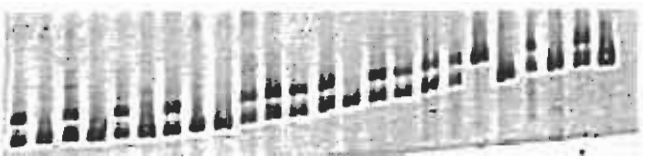
Marker	Marshfield map position (cM)	LOD scores at θ								
		0.0	0.01	0.05	0.1	0.2	0.3	0.4	Zmax	θ max
D6S276	44.41	-∞	2.55	2.91	2.73	1.97	1.05	0.24	2.91	0.05
D6S291	49.50	-∞	1.32	2.33	2.43	1.95	1.21	0.48	2.43	0.10
D6S1610	53.81	2.44	2.39	2.17	1.88	1.26	0.66	0.21	2.44	0.0
D6S1019	53.81	3.29	3.24	3.01	2.68	1.95	1.17	0.46	3.29	0.0
D6S1017	63.28	-∞	2.14	3.00	2.93	2.19	1.27	0.47	3.00	0.05

of 2.93 at recombination fraction 0.1 resulted from two-point analysis for marker D6S1017. Other proximal markers D6S291 spaced at 4.31 cM and D6S276 spaced at 5.09 cM from D6S1019 yielded 2.43 lod score at recombination fraction 0.10 and 2.91 lod score at recombination fraction 0.05, respectively. Lod score analysis indicates that the region between D6S276 and D6S1017 with a distance of 18.87 cM is the disease region.

All the affected individuals are homozygous for markers D6S291, D6S1610, and D6S1019. The affected members III-6, III-8, III-9, IV-5, IV-11, IV-13, IV-18, V-1, V-11 and V-12 are homozygous for all the markers. Affected member IV-8 is heterozygous for marker D6S276. Affected member IV-19 is heterozygous for marker D6S1017. Individual V-3 is heterozygous both at markers D6S276 and D6S1017. Recombination events involving markers D6S276 and D6S1017 define the telomeric and centromeric boundries, respectively of the disease region.

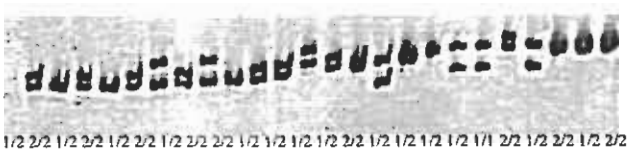
Recombination at markers D6S276 and D6S291 was seen in members III-7, and V-3 whereas, IV-8 showed the transmission of maternal cross over. In affected members, IV-19 and V-3 recombination at markers D6S1019 and D6S1017 was observed. Strong consanguinity is observed in this family of blinds. All marriages were close relative marriages. Due to these consanguineous marriages most of the normal members are carriers for blindness allele.

This study revealed the presence of disease region at 6p21.2. At this region (6p21.2) a candidate gene RDS is present, and a candidate gene TULP1 is also present in close approximity at 6p21.3. In order to exclude one of the candidate genes screening markers D6S1017 for RDS (Ali *et al.*2000; Dryja *et al.*1997; Farrar *et al.* 1991; Felbor *et al.*1997) and D6S1281 for TULP1 (Banerjee *et al.*1998; Hagstrom *et al.* 1998; Knowles *et al.*1994; Hagstrom *et al.*2001) were typed. Fig.14 represent the gel photograph of markers, which exclude one of the candidate genes, RDS (D6S1017) and TULP1 (D6S1281). Alleles were assigned to the individuals and their haplotypes are shown in the Fig.15. Microsatellite marker D6S1017 showed significant linkage with crossovers in 2 affected individuals (IV-19 and V-3), whereas all other affected individuals were homozygous (III-6, IV-5, IV-8, IV-11, IV-13, IV-18, V-1, V-11, and V-12).



2/3 3/3 2/3 3/3 2/3 1/3 2/3 1/3 3/3 2/3 2/3 ½ 2/3 3/3 3/4 3/3 3/3 3/4 3/4 2/3 3/4 3/3 3/3 3/3

D6S1281



1/2 2/2 1/2 2/2 1/2 2/2 1/2 2/2 2/2 1/2 1/2 1/2 1/2 2/2 1/2 1/2 1/2 1/2 1/1 2/2 1/2 2/2 1/2 2/2

D6S1017

Fig.14. Screening markers used for the presence of Tulp1 (D6S1281) or RDS/peripherin gene (D6S1017), in 7CBL family.

The microsatellite marker D6S1281 showed loose linkage with crossovers in 4 affected individuals (IV-5, IV-8, V-3 and V-11), whereas all other affected individuals were homozygous (III-6, IV-11, IV-13, IV-18, IV-19, V-1 and V-12) (Fig. 15).

Taking in view the homozygosity mapping and significant linkage with marker for RDS, the possibility of location of macular degeneration disorder in this family is in the region of gene RDS than TULP1 gene. In digenic RP, 6p21.2 region is also involved with another locus on chromosome 11 (Dryja *et al.*1997; Kajiwara *et al.*1994), therefore we also analyzed markers of chromosome 11 of digenic RP region (D11S1313, D11S1985, D11S4191) in order to check the possibility of digenic RP (Fig.16). From haplotype analysis (Fig. 17) it is evident that these markers showed no linkage, thus excluding the possibility of digenic RP and suggesting the presence of RDS candidate gene for macular degeneration in the family studied.

Discussion7CBL

In this study a Pakistani consanguineous family suffering from congenital blindness was diagnosed for autosomal recessive macular degeneration. Linkage studies revealed the presence of disease locus at 6p21.2 where a candidate gene peripherin/RDS for eye diseases is present, which is involved in dominant RP, dominant macular degeneration, digenic RP with ROM1 and adult vitelliform macular degeneration (Ali *et al.* 2000; Dryja *et al.*97; Felbor *et al.*1997; Kajiwara *et al.* 1994). Two point lod score gave maximum value of 3.29 at recombination fraction 0 for microsatellite marker D6S1019 and lod score of 3.0 at recombination fraction 0.05 for microsatellite marker D6S1017 which is a tightly linked marker of RDS gene (Ali *et al.* 2000; Dryja *et al.*1997; Farrar *et al.* 1991; Felbor *et al.* 1997).

Macular dystrophies are usually inherited in a predictable pattern within families. There is genetically determined abnormality causing either the loss of the normal pigment associated with the photoreceptors in the retina or the accumulation of an abnormal protein. The age of onset is variable, but mostly appear in the first two decades of life (Prevost, 2000; Michaelides *et al.* 2003a).

There is considerable clinical and genetic heterogeneity: macular dystrophies showing autosomal dominant, autosomal recessive, X-linked recessive and mitochondrial inheritance have all been reported (Michaelides *et al.* 2003a). Present study shows an

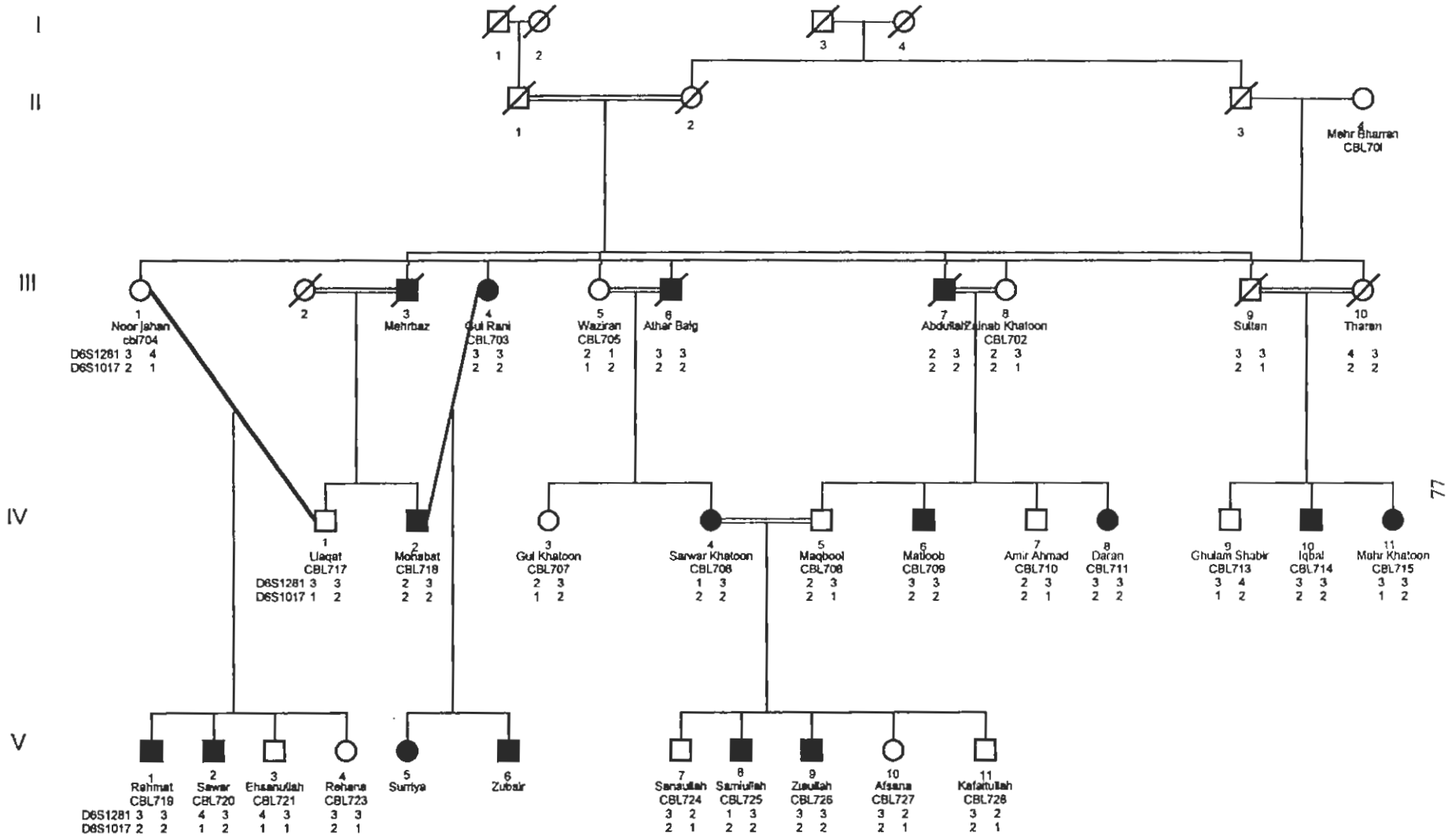
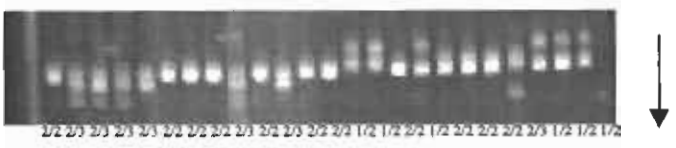
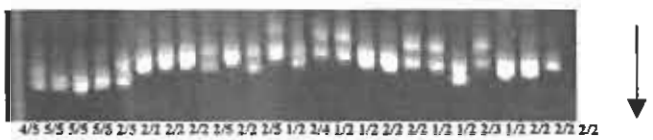


Fig.15. Part of Pedigree 7CBL (numbering of individuals as in Fig.11) showing haplotypes of individuals searched for TULP1or RDS gene. Haplotypes of III-8, III-9, III-11 and III-12 are assumed from progeny



D11S1313



D11S1985

D11S4191

Fig.16: Markers, D11S1313, D11S1985 and D11S4191 used for the exclusion of digenic RP (presence of ROM1 with RDS), in the family 7CBL.

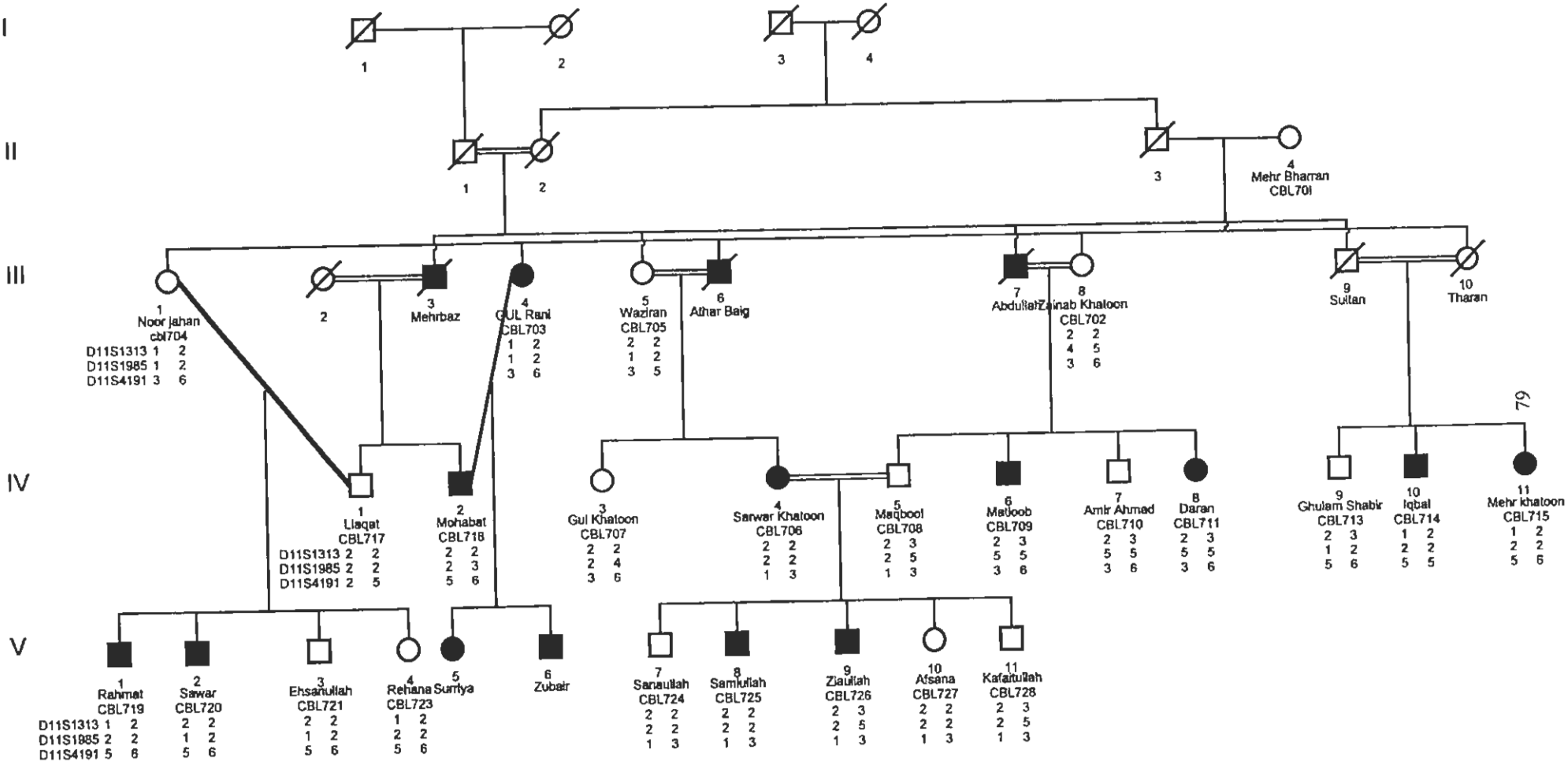


Fig.17 Part of pedigree 7CBL (numbering of individuals as in Fig.11), Haplotypes of individuals showing the exclusion of digenic RP.

autosomal recessive mode of inheritance for macular degeneration with congenital onset of the disease. The maculae were degenerated and pigmented with a rim of chorioretinal atrophy.

Classification of macular degeneration is more tenuous (Fishman, 1990). Macular degeneration can have either a genetic basis or it may be an acquired disease. Approximately 10% of Americans over the age of 50 are afflicted with age-related macular degeneration (Bressler *et al.*1988), an acquired form of disease. The inherited forms of macular degeneration are much less common but usually more severe. The inherited forms are characterized by early development of macular abnormalities such as yellowish deposits and atrophic or pigmented lesions, followed by progressive loss of central vision. The macular lesions may appear early in life and often precede the loss of vision by many years (Daiger *et al.* 1995). Like retinitis pigmentosa, macular degeneration is usually bilateral. Unlike retinitis pigmentosa, only the central retina is affected. The relative incidence and importance of inherited versus non-inherited factors is unclear. In addition, genetic factors are likely to play a role in acquired forms of macular degeneration. The high frequency of acquired forms makes it very difficult to determine the incidence of inherited forms. However, several distinct, heritable forms of the disease such as autosomal dominant North Carolina macular dystrophy, autosomal dominant Best macular dystrophy, and autosomal recessive Stargardt disease have been mapped (Daiger *et al.* 1995).

Stargardt macular dystrophy (STGD) is the most common inherited macular dystrophy with a prevalence of 1 in 10,000 with an autosomal recessive mode of inheritance. It shows a very variable phenotype with a variable age of onset and severity. Most cases present with central visual loss in early teens and there is typically macular atrophy with white flex at the level of the retinal pigment epithelium (RPE) at the posterior pole on Ophthalmoscopy. The locus for STGD/FFM was mapped to chromosome 1p using homozygosity mapping in inbred families, and the causative gene identified was ABCR (Fishman *et al.*, 1987). ABCR encodes a transmembrane rim protein located in the disc of rod and foveal cone outer segments, that is involved in ATP dependent transport of retinoids from photoreceptor to RPE (Sun and Nathans, 1997;

Molday *et al.* 2000; Weng *et al.*1999). Failure of this transport results in deposition of a major lipofuscin fluorophore, A2E (N-retinylidene-N-retinylethanolamine), in the RPE (Weng *et al.*, 1999). It is proposed that this accumulation may be deleterious to the RPE, with consequent secondary photoreceptor degeneration. Subsequently, mutations in ABCR have been implicated in other disorders , including retinitis pigmentosa (Martinez *et al.*, 1997; Martinez *et al.*, 1998) and cone-rod dystrophy (CORD) (Cremers *et al.*, 1998; Maugeri *et al.*, 2000). In the present study the autosomal recessive macular degeneration is clinically in agreement with the Stargardt macular dystrophy (STGD) but it showed a linkage at chromosome 6 with microsatellite markers D6S276, D6S291, D6S1610, D6S1019 and D6S1017. The locus mapped in this study was at 6p21.2, where RDS gene is involved in the development of the disease.

Genes causing the inherited forms of macular degeneration (MD) are very promising candidates for genetic factors that predispose to age related-macular degeneration (Daiger *et al.*1995). The first mapped gene for autosomal dominant MD was VMD1(vitelliform macular dystrophin 1), assigned to 8q (Ferrell *et al.* 1983). Subsequently, the gene for North Carolina macular dystrophy was mapped to 6q (Small *et al.* 1992) and the gene for Best macular dystrophy was mapped to 11 q (Stone *et al.* 1992a). The gene for autosomal recessive STGD, was mapped to chromosome 1p (Kaplan *et al.*1993), two genes for autosomal dominant STGD were mapped to 6q14 and 4p (Stone *et al.* 1994; Kniazeva *et al.*1999), RDS gene for autosomal dominant pattern dystrophies was mapped on 6p (Farrar *et al.* 1991; Kajiwara *et al.* 1991), EFEMP1 gene (that code EGF-containing fibrillin-like extracellular matrix protein 1) for autosomal dominant drusen was mapped on 2p (Stone *et al.*1999), the gene for autosomal dominant drusen and macular degeneration was mapped on 6q14 (Kniazeva *et al.* 2000), autosomal dominant progressive bifocal chorioretinal atrophy has been linked to 6q14-q16.2 (Kelsell *et al.* 1995),TIMP3 gene for Sorsby fundus dystrophy was mapped to 22q (Weber *et al.*1994), and XLRS1 gene for x-linked juvenile retinoschisis was mapped to Xp22.2 (Sauer *et al.*1997). Additional genes causing autosomal dominant MD have been mapped to 6p and 7p (Kremer *et al.* 1994). The gene for autosomal recessive was reported at 1p by Kaplan *et al.* 1993, whereas in the present study the disease locus was observed at 6p21.2 for autosomal recessive macular degeneration which was a previously

reported locus for autosomal dominant macular degeneration (Farrar *et al.* 1991; Kajiwara *et al.* 1991).

Peripherin/retinal degeneration slow (RDS) is a membrane-associated glycoprotein found in the outer segments of retinal rod and cone photoreceptor cells. It is thought to play a role in membrane srtuctural stabilization, in conjunction with retinal outer segment membrane protein 1 (Payne *et al.* 1998). They performed mutation analysis by heteroduplex and direct sequencing of PCR-amplified coding exons of the RDS gene in 300 British patients with dominantly inherited macular dystrophies. They found that 7.3% of this group had peripherin/RDS mutations, segregating with disease. They concluded that the most commonly occurring peripherin/RDS mutations in British population was 172ArgTrp mutation involved in cone dystrophy, macular dystrophy, and central areolar chorodial dystrophy. Present study also revealed the involvement of RDS gene in autosomal recessive macular degeneration.

Mutations in the RDS gene give rise to retinal degenerations with a wide phenotypic spectrum. The majority of mutations result in macular dystrophies (Keen and Inglehearn 1996). Specific mutations in the RDS gene may lead to a wide inter and intrafamilial variability of phenotype, as seen in one family with retinitis pigmentosa, pattern dystrophy, and fundus flavimaculatus, in three different members with a deletion at codon 153/154 (Weleber *et al.* 1993). Present study suggests that there may be some mutations in RDS gene which may be responsible for autosomal dominant as well as autosomal recessive macular degeneration.

Felbor *et al.* (1997) found mutations in the peripherin/RDS gene, which encodes a photoreceptor-specific membrane glycoprotein in a variety of retinal phenotypes. However, they were of the view that mechanisms by which specific mutations in this gene can cause typical features of retinal dystrophies clinically as distinct as retinitis pigmentosa or macular degeneration are still unknown. Recently, a single case of adult vitelliform macular dystrophy (AVMD) has been associated with a Y258Stop mutation. To assess the frequency of peripherin/RDS mutations in the clinically heterogeneous group of AVMD, they analyzed the entire coding region of the gene in 28 unrelated patients. They identified five novel mutations including two presumed null allele mutations. Thus, their results demonstrate that a significant portion

of AVMD patients (18%) carry point mutations in peripherin/RDS, suggesting that this gene is frequently involved in the pathogenesis of this macular disorder. In addition, their study shows that the variable phenotypes in AVMD are due, at least in part, to genetic heterogeneity and are likely to be caused by mutations in disease genes thus far unknown. RDS gene for autosomal dominant pattern dystrophies was mapped on 6p (Farrar *et al.* 1991; Kajiwara *et al.* 1991). Present study mapped the locus for autosomal recessive macular degeneration at 6p21.2, which was a previously reported locus for autosomal dominant macular degeneration from non-Asian populations (Farrar *et al.* 1991; Kajiwara *et al.* 1991). It is suggested that mutation at this locus might have taken place resulting in recessive form of macular degeneration or this is just possible that in this family from Pakistan this disorder may be controlled by recessive gene. These results suggest that inheritance for macular degeneration could be genetically hetrogeneous (Michaelides *et al.* 2003a).

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RESULTS
CHAPTER-3

INTRODUCTION

Fundus Dystrophy:

Sorsby's fundus dystrophy (SFD) is an autosomal dominant retinal degeneration in which patients first complain of night blindness, then experience central visual loss from subretinal neovascularization and haemorrhage, and later loss of peripheral vision (Sorsby *et al.* 1949, Hoskin *et al.* 1981, Capon *et al.* 1988, Polkinghorne *et al.*1989, Hamilton *et al.* 1989 and Wu *et al.* 1991). SFD is a relatively rare disease but it commands considerable interest because it is the only genetic disease in which haemorragic macular degeneration occurs in the majority of affected patients. Haemorrhagic macular degeneration also occurs in age-related macular degeneration (ARMD), the leading cause of blindness in the Western world in individuals over fifty years of age (Bressler *et al.* 1988).

Although a exact pathophysiology in SFD remains unknown, some clues have been gathered. One clue came from morphological study of a retina from an eye donor with SFD which showed an abnormal lipid-containing deposit in interposed between the photoreceptor and their blood supply, the choroid (Capon *et al.* 1989). This subretinal deposit, located within Bruch's membrane and present across the entire retina, was suggested as a possible barrier to diffusion of nutrient to the photoreceptors (Steinmetz *et al.* 1992). More recently, the disease causing gene in two SFD families was discovered to be tissue inhibitor of metalloproteinases-3 (TIMP3) and it was speculated that mutations could lead to the abnormal subretinal deposit by disturbing the balance between buildup and breakdown of the extracellular matrix (Weber *et al.* 1994).

The SFD locus was initially localized to chromosome 22q13-ter (*Weber et al.*1994a) and subsequently refined to an 8-cM interval between loci D22S275 and D22S278 (Gregory *et al.* 1995). Two mutations, Tyr168Cys and Ser181Cys, of the gene encoding tissue inhibitor of metalloproteinases-3 (TIMP3), also mapping to 22q12.1-q13.2 (Apte *et al.*1994), have been shown to segregate with disease in two separate North American SFD pedigrees (Weber *et al.* 1994b). Felbor *et al.* 1995 described new SFD families with mutations in TIMP3 at codon Ser156Cys and Jacobson *et al.*1995 described Gly167Cys mutation in TIMP3. TIMP3 is present in the extracellular matrix of various

tissues and the presence of a TIMP-like protein has been reported in the inter photoreceptor matrix (Steinmetz *et al.* 1992). In SFD the TIMP3 protein may be directly or indirectly responsible for the deposition of material at the level of Bruch's membrane, which may subsequently lead to the manifestation of the SFD phenotypes.

The aim of the present study is to search any new locus for this disorder and how consanguinity influences in the appearance of this disorder. These contributions in this regard may be helpful in future research regarding gene therapy of this disorder.

RESULTS

EPIDEMIOLOGY

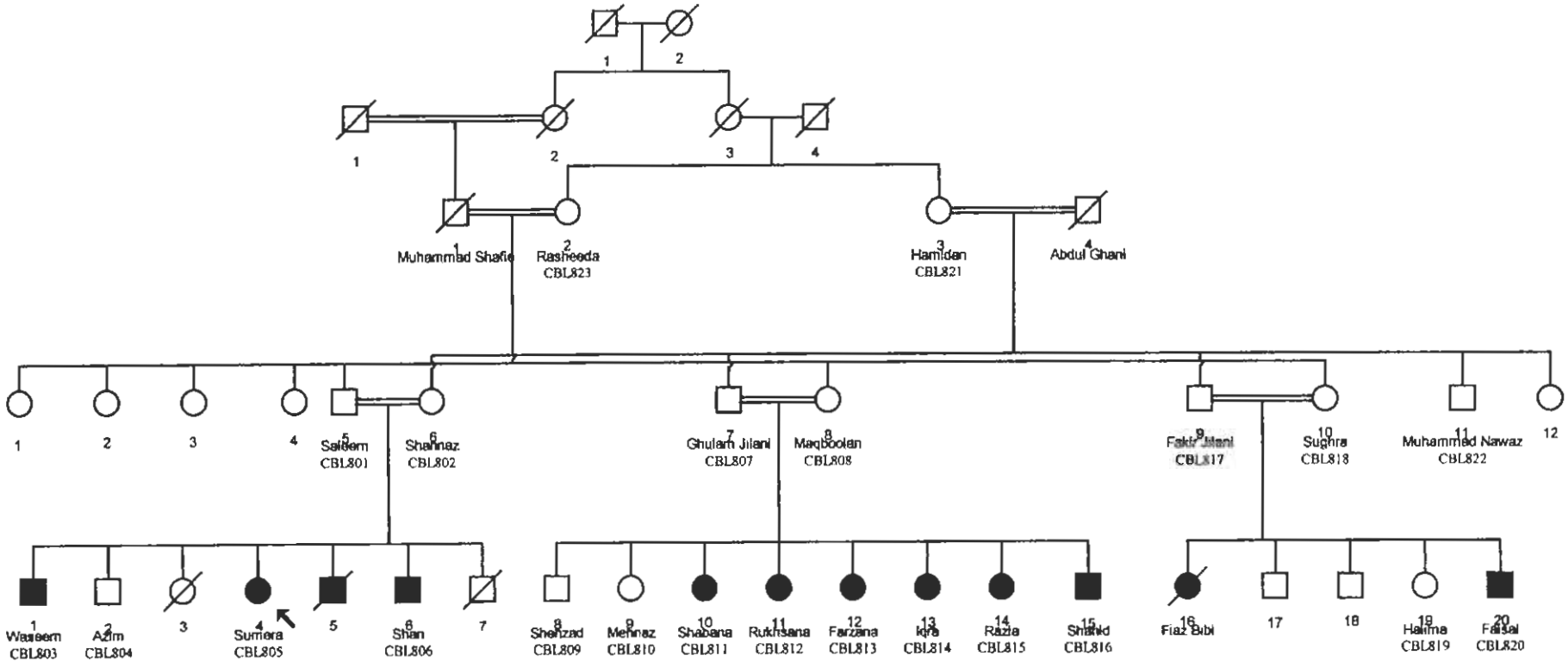
A Pakistani consanguineous kindred suffering from congenital blindness was identified (Fig.18). The surname of the family is Rajput. The kindred migrated from Amritsar (India) and currently they are settled in the District Jhang and the District Faisalabad. They are very poor and un-educated. The affected children are studying in the Special Education Schools. The normal individuals of the kindred were healthy, both physically and mentally. The affected members bear meningial bulging with a patch of alopacea about 1-1.5cm in diameter in the occipital region (Fig.19). However, no other physical and mental disability was observed in the affected members except V-1 who was epileptic.

In this kindred mostly consanguineous marriages were contracted, resulting in a higher number of affected children. Of the live members, 12 were affected including five males and seven females.

Individual V-4 is the proband who was first diagnosed for the ocular disorder and with her help the disorder was traced back in the family.

In the first generation distantly related normal couple (I-1 and I-2) produced two normal daughters (II-2 and II-3).

In the second-generation first cousin marriage was contracted between (II-1) and (II-2) both normal for blindness and produced one normal son (III-1). Individual II-3 was married to normal man (II-4) and produced two normal daughters (III-2 and III-3).In the third generation both marriages were between first cousins. Marriage between III-1 and III-2, both normal for this disorder, produced six normal daughters (IV-1, IV-2, IV-3, IV-4, IV-8 and IV-10) and one normal son (IV-5). Another first cousin marriage between



.18. Pedigree of a consanguineous Pakistani family suffering from autosomal recessive hereditary fundus dystrophy, 8

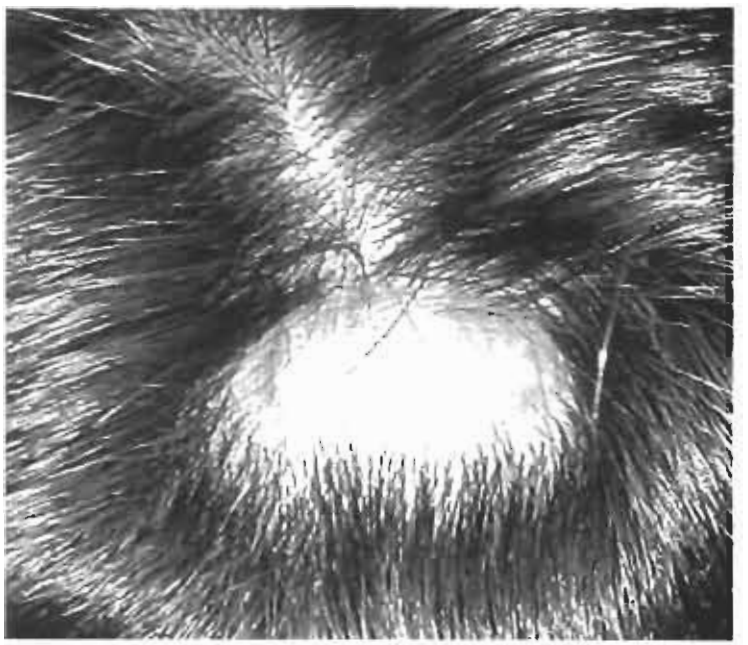


Fig.19. Photograph of an affected individual (V-4) illustrating Meningial bulging with alopacea in the occipital region.

III-3 and III-4 both normal, produced two normal daughters (IV-6 and IV-12) and three normal sons (IV-7, IV-9 and IV-11).

In the fourth generation there were three first cousin marriages. All of them were normal for blindness. One marriage was between (IV-5) and (IV-6) who produced three affected sons (V-1, V5 and V-6), two normal sons (V-2 and V-7), one affected daughter (V-4) and one normal daughter V-3. Another first cousin marriage was between (IV-7) and (IV-8) who have one normal son (V-8), one normal daughter (V-9), five affected daughters (V-10, V-11, V-12, V-13, and V-14) and one affected son (V-15). Third first cousin marriage was between (IV-9) and (IV-10) they have one affected daughter (V-16), one normal daughter (V-19), two normal sons (V-17 and V-18) and one affected son (V-20).

In this kindred mode of inheritance for this ocular disorder was ascertained as autosomal recessive. The family was diagnosed for hereditary fundus dystrophy.

Clinical Assessment:

Four affected members of the family 8CBL (V-4, V-6, V-11, and V-12) were clinically examined. Their ages were 12 years, 9years, 15years and 9 years respectively.

Age of onset:

All the affected members of the family were congenitally blind.

Parent's observations:

Parents of the blind children noted meningial bulging with a patch of alopacea in the occipital region of the blind patients and they reported that any of their siblings who had this patch turned out to be blind whereas, their normal siblings had no such phenotype. The patch was about 1 to 1.5cm in diameter, indicating occipital pole of the brain is degenerated (Fig.19).

Clinical description:

All four patients examined have nystagmus of various degrees. The visual acuity varied from 20/400 to no perception of light. V-4 and V-11 had sublaxated lens with cataract formatin. The cataract was zonular in type. Individual V-11 has had one eye operated, which had no perception of light. She developed glaucoma (absolute eye) with marked vascularization of iris. There was also haemoragic eye in the viterous. The

second eye showed sublaxation. Individuals V-6 and V-12 also showed sublaxation of lens. Anterior segments were normal. Pupil reaction to light was diminished.

Fundus:

In all affected individuals the discs were within normal limits in color but there was peripapillary degeneration. Veins were normal in size. Arterioles showed some attenuation. Macula showed degenerative changes. There was a blackish pigment spot in the macular region and also whitish grey fibrosis reaction. The black spot may be haemoragic eye spot. Periplony revealed only minimal pigmentary changes (Fig.20). Most significant change was marked choroidal vascular degenerative changes.

Associated abnormalities:

Any associated abnormality like polydactly, obesity, hypogonadism, hearing loss, mental retardation and skeletal abnormalities was not found except ocular disorder. Only one affected individual V-1 was suffered from neurological disorder.

Diagnosis:

The affected individuals were diagnosed for hereditary fundus dystrophy.

Molecular studies:

The affected members of this family were diagnosed for a distinct phenotype of fundus dystrophy with meningial bulging having a patch of alopacea in their occipital region. Twenty members of this family were sampled for molecular studies. Known loci for leber congenital amaurosis were first excluded by linkage analysis, using locus specific polymorphic microsatellite markers (Table-8; pp. 43-44). Subsequently a genome wide search was carried out using a set of 397 polymorphic microsatellite markers spanning the entire genome at ~10-15cM intervals (Research Genetics, version-8).

A hint of linkage was observed at 22q12.1-q13.2 with microsatellite markers D22S280 and D22S685 through homozygosity mapping. The microsatellite markers which were searched in this region were D22S280, D22S685, D22S278, D22S283, D22S423, and D22S282 (Fig.21).

Alleles were assigned to the individuals and their haplotypes are shown in the Fig.22. In one branch the affected members (V-10, V-11, V-13, V-14 and V-15) showed

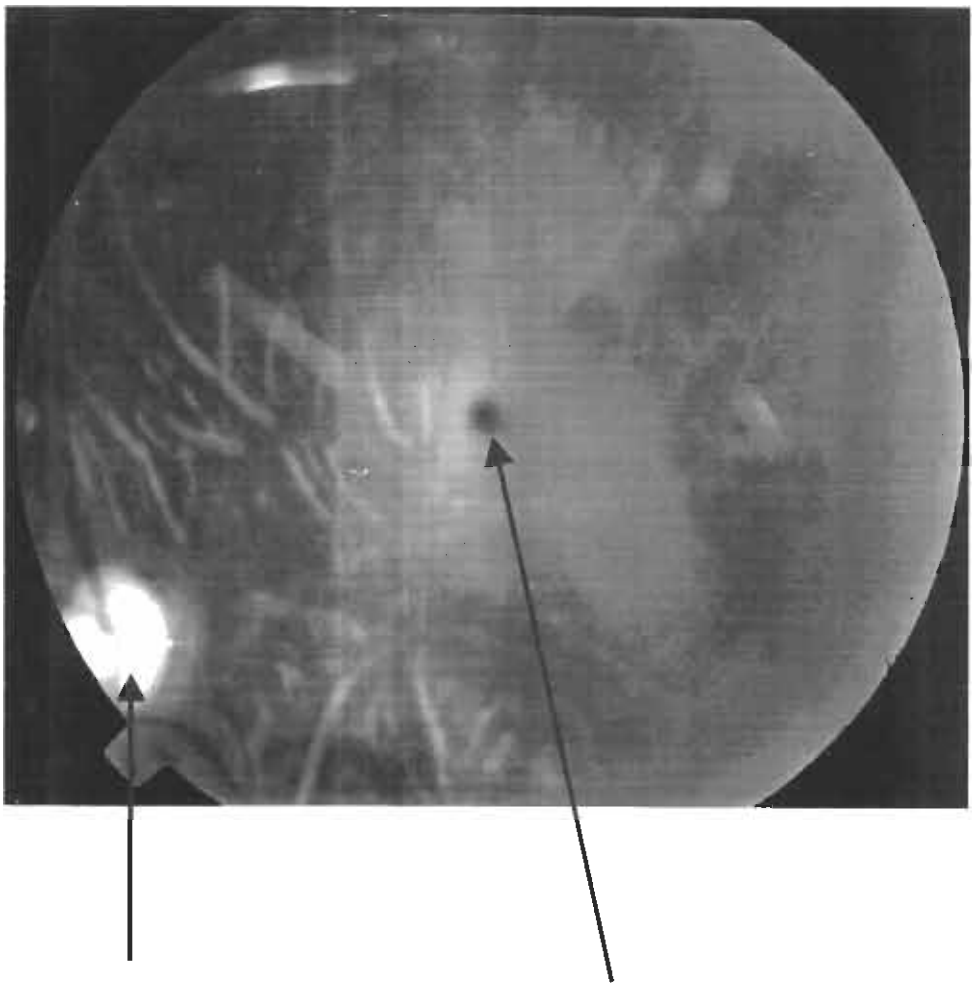
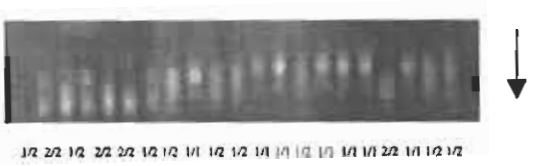
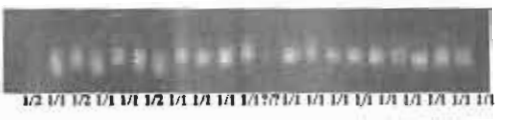


Fig.20: Fundus of an affected individual.



DS22S280

D22S685



D22S278

D22S283

D22S423

D22S282

Fig.21. Gel photograph of themarkers,D22S280, D22S685, D22S278, D22S283, D22S423 and D22S282 used in the family 8CBL.

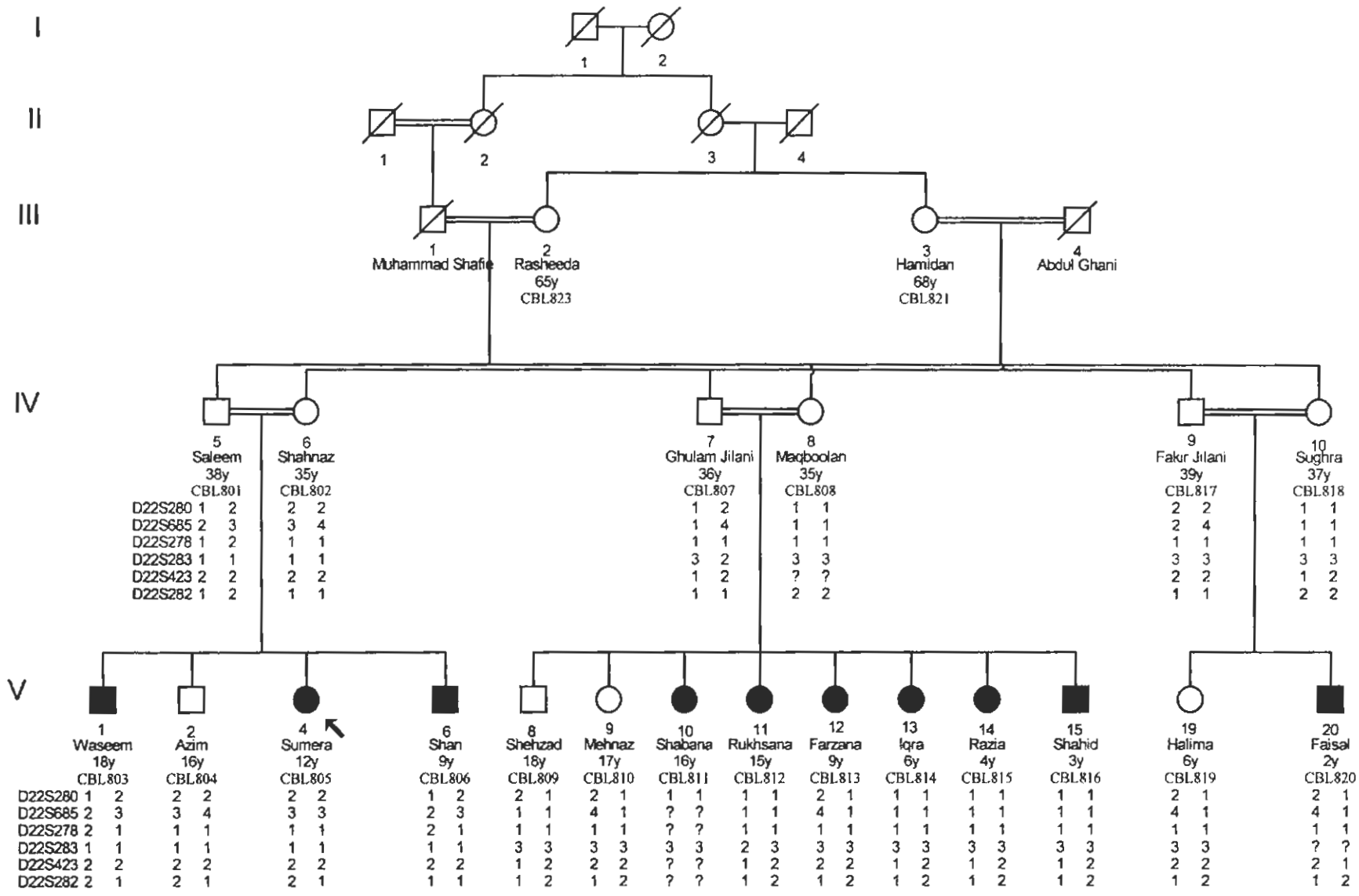


Fig.22. Part of pedigree 8CBL (numbering of individuals as in Fig.18) showing haplotypes of individuals.

homozygosity (1/1) for markers D22S280 and D22S685 with the exception of affected individual V-12 who is heterozygous (2/1 and 4/1) with both markers. The affected individuals come from a marriage between IV-7 and IV-8. All the affected individuals, inherited haplotype 1 1 1 3 2 2 from their mother (IV-8). The other haplotype in affected individuals, V-13, V-14 and V-15 is 1 1 1 3 1 1. Affected individual V-11 inherited 1 1 1 2 1 1 haplotype from father, this appears that crossing over in the paternal haplotype has taken place between D22S278 and D22S283. Haplotype of affected individual V-10 is difficult to explain due to non amplification of markers. Affected individual V-12 inherited 2 4 1 3 2 1 which resulted in crossing over between D22S278 and D22S283 and also between D22S283 and D22S423. In normal individual, V-9, the haplotypes are similar to the affected individual V-12 (2/1 4/1 1/1 3/3 2/2 1/2) which is difficult to explain at the moment. It is quite possible that in this individual which bears genotype like an affected individual the onset may be later.

Haplotype analysis represents the disease region between D22S280 and D22S283, which is 7.32 cM apart. The distance between markers D22S280 and D22S685 is 1.09 cM, between D22S685 and D22S278 is 3.83 cM and between D22S278 and D22S283 is 2.40 cM (Fig. 22).

Two other branches of the pedigree, one offspring coming from consanguineous marriage between IV-5 and IV-6 and the other coming from consanguineous marriage between IV-9 and IV-10 are difficult to explain. The affected individuals coming from marriage between IV-5 and IV-6, have a different pattern of homozygosity, than in the offspring from marriage between IV-7 and IV-8. One affected individual V-4 shows homozygosity at D22S280 (2/2), D22S685 (3/3) and D22S278 (1/1). Other affected individuals V-1, and V-6 gave no conclusive hint of linkage, therefore, this branch of family showed no hint of linkage at chromosome 22. In the other consanguineous marriage (IV-9 and IV-10) both affected (V-20) and normal individual (V-19) are heterozygous at D22S280 and D22S685. Again this branch showed no hint of linkage in this region.

During whole genome search it was seen that one branch (consisting of products of consanguineous marriage between IV-5 and IV-6) showed a hint of linkage at chromosome 8 with marker D8S592, while other two branches showed no hint of linkage

in this region (Fig.23). This branch showed homozygous haplotype (2/2) with microsatellite marker D8S592 in all affected individuals (V-1, V-4 and V-6) and heterozygous haplotype in normal individual V-2. Due to homozygosity mapping mapping, hint of linkage was observed at this locus but we cannot calculte the lod score due to less number of informative meioses in this branch.

The other two branches of the family coming from a marriage between IV-7 and IV-8, and between IV-9 and IV-10 showed no clue of linkage with the marker D8S592. As both the affected and normal individuals in the both branches showed the same haplotype.

It appears in these affected individuals the presence of disease may be due to consanguinity. These individuals show homozygosity at different markers. It may be assumed that homozygosity at different positions may be due to the heterogeneity of the disease

Discussion 8CBL:

In this study a Pakistani consanguineous family suffering from autosomal recessive congenital blindness was diagnosed with distinct phenotype. The affected individuals bear meningial bulging with a patch of alopacea about 1-1.5cm in diameter in the occipital region. Such distinctive phenotype was not previously reported. All the affected individuals have complex ocular disorders having nystagmus, sublaxated lens with zonular type of cataract, peripapillary degeneration, marked choroidal changes and haemoragic eye spot.

Homozygosity mapping revealed the presence of disease locus at chromosome 22q12.1-q13.2, which is a previously reported locus for dominant Sorsby's fundus dystrophy (Felbor *et al.*1995; Felbor *et al.* 1997; Jacobson *et al.* 1995; Stohr *et al.*1995 and Weber *et al.* 1994). The microsatellite markers D22S280 and D22S685 showed hint of linkage with one branch through homozygosity mapping, while other two branches show homozygosity at different markers. It appears in these affected individuals the presence of disease may be due to consanguinity. It may be assumed that homozygosity at different positions may be due to the heterogeneity of the disease.

Sorsby fundus dystrophy (SFD) was described by Sorsby and Mason, in 1949, as an autosomal dominant disorder in which patients experience a rapid decline of central

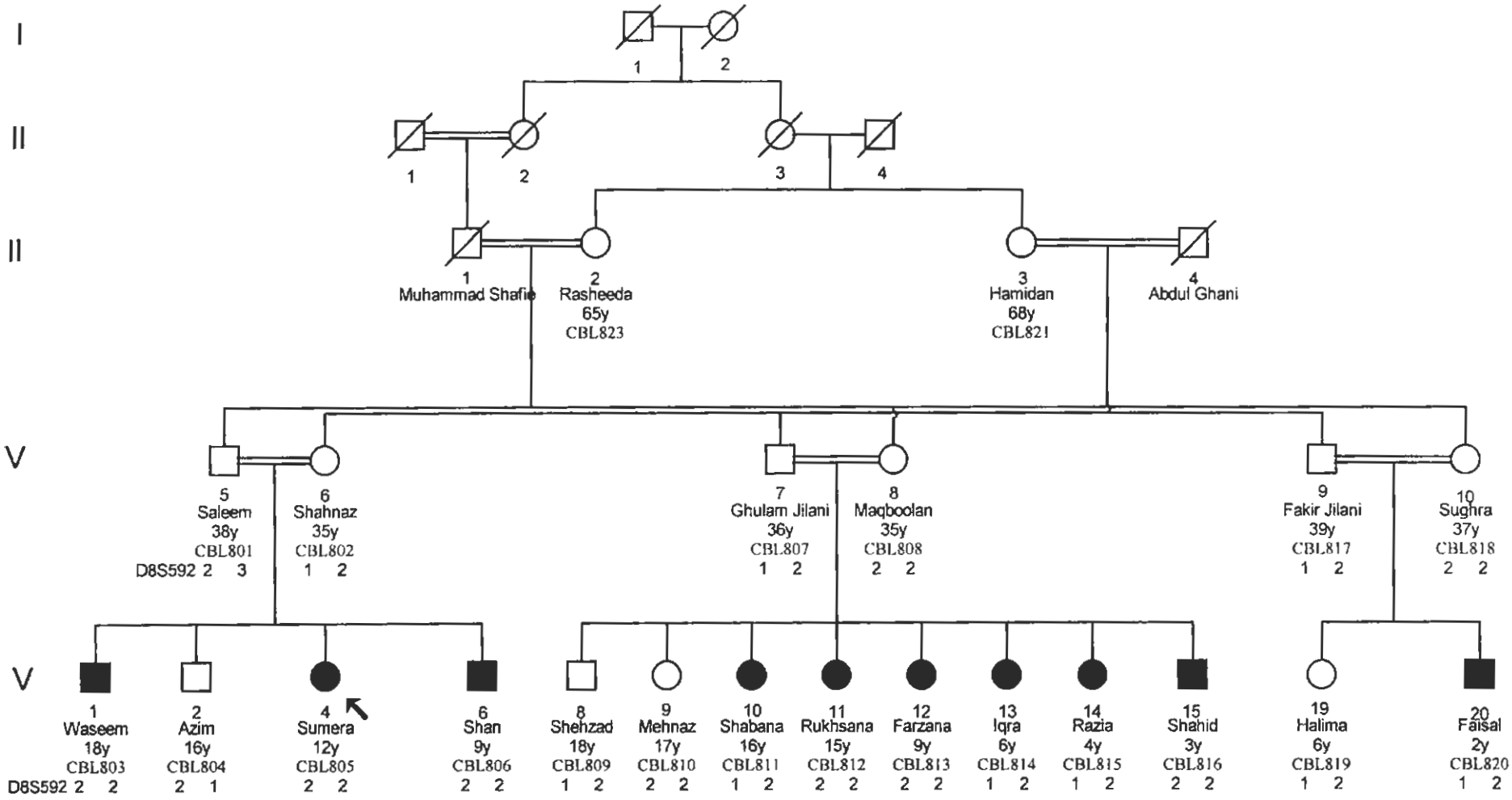


Fig.23 Part of Pedigree 8CBL (numbering of individuals as in Fig.18) showing haplotypes of individuals.

vision during their 40s and lose ambulatory vision 3-4 decades later. In his original report, Sorsby noticed bilateral macular "hemorrhage and exudates developing generalized choroidal atrophy with massive pigment proliferation" (Sorsby *et al.* 1949). Weber *et al.* (1994) mapped the gene for Sorsby fundus dystrophy to 22q13-qter, and examined that the TIMP3 gene as a possible site of causative mutations in SFD on the basis of its chromosomal location and its pivotal role in extracellular matrix remodeling. They identified point mutations in TIMP3 in affected members of 2 SFD pedigrees. These mutations were predicted to disrupt the tertiary structure and thus the functional properties of the mature protein.

Weber *et al.* (1994) described that mutations in TIMP3 have been shown to cause SFD, an autosomal dominant disorder of the macula with the onset in the third or fourth decade of life. In the present study the family showed an autosomal recessive mode of inheritance and early manifestation of fundus dystrophy or hereditary congenital fundus dystrophy with a distinct phenotype (meningial bulging with alopacea in the occipital region of all blind individuals). It is suggested that due to consangunity and a recessive mutation other than the reported dominant TIMP3 mutation has caused congenital blindness as well as some unknown mechanism, which is involved in the appearance of alopacea in the occipital region of all blind individuals. Present study showed a hint of linkage with marker D22S280 and D22S685, which are, linked markers of Sorsby's fundus dystrophy and showed the involvement of TIMP3 in distinct phenotype of congenital blindness. Mutation screening is in progress to see the exact type of mutation in TIMP3, other than reported in Sorsby's fundus dystrophy. Mutations in TIMP3 gene have been associated exclusively with the SFD phenotype (Weber *et al.* 1994; Felbor *et al.* 1996; Felbor *et al.* 1995 and Jacobson *et al.* 1995). It appears from above studies that only a single type of mutation affecting the C-terminus of the protein causes the clinical features of SFD, because all known mutations have been identified in exon five of the TIMP3 gene and lead to an additional cysteine residue in the mature protein. This raises the possibility that another type of mutation in TIMP3 qualitatively different from the known one could result in a clearly distinct phenotype.

Stohr *et al.* (1995) reported the genomic organization of the TIMP3 gene. TIMP3 is encoded by 5 exons extending over approximately 55 kb of genomic DNA. They

compared the 5-prime flanking sequences of the human and mouse TIMP3 genes and found a high degree of similarity between them. By hybridization to a panel of human/hamster somatic cell hybrid DNAs, Apte *et al.* (1994) mapped the TIMP3 gene to chromosome 22; by in situ hybridization, they regionalized the assignment to 22q12.1-q13.2. By analysis of a panel of mouse/human hybrids, Wilde *et al.* (1994) assigned the gene to chromosome 22. Our linkage studies also showed a hint on chromosome 22.

In this study the disease was mapped at locus 22q12.1-q13.2 through homozygosity mapping with microsatellite markers D22S280 and D22S685. The disease is recessively controlled in this family. The locus identified in this study is the same as has been reported for dominantly controlled Sorby's fundus dystrophy. The symptoms indicated in clinical examination are similar to that of Sorby's fundus dystrophy except alopacea in the occipital region. Present disease seems to be related to Sorsby's fundus dystrophy but is recessively controlled. It is suggested that in affected individuals TIMP3 gene is involved in the development of this disorder. Whole genome search also showed a hint of linkage at chromosome 8 with marker D8S592 which is an indication of genetic heterogenity within the same closely inbred family. This study suggests that two different loci one at chromosome 8 and other at chromosome 22 are controlling the development of the disease in this family.

RESULTS
CHAPTER-4

INTRODUCTION

Inherited retinal diseases are a common cause of visual impairment in children and young adults, often resulting in severe loss of vision in later life. The most frequent form of inherited retinopathy is retinitis pigmentosa (RP), with an approximate incidence of 1 in 3,500 individuals worldwide (Sullivan *et al.*1999). RP is characterized by night blindness and progressive degeneration of the midperipheral retina, accompanied by bone spicule-like pigmentary deposits and a reduced or absent electroretinogram (ERG). The disease process culminates in severe reduction of visual fields or blindness. RP is genetically heterogeneous, with autosomal dominant, autosomal recessive and X-linked forms. RP may be non-syndromic or syndromic.

Non-syndromic:

Prevalence of non-syndromic retinitis pigmentosa is 1/4,000 (Ammann *et al.*1965; Boughman *et al.*1980; Jay, 1982). The clinical features of retinitis pigmentosa vary among patients and even among family members with the disease. Typical RP causes death of rod cells first and results in night blindness and "tunnel vision" from loss of peripheral vision. Central vision in RP usually is spared until later age. The age of onset varies with the type of RP but generally begins by young adult years and advances slowly over decades to cause massive vision impairment or loss by late age.

Mode of Inheritance :

Retinitis pigmentosa may occur as an isolated disorder, inherited in an autosomal dominant, autosomal recessive, X-linked, or mitochondrial manner or it may occur in association with certain systemic disorders, which are usually autosomal recessive in inheritance. Rare digenic forms also occur.

Autosomal Dominant RP

Autosomal dominant RP is the most common, accounting for 43% of cases. Autosomal dominant forms are usually the mildest forms, with some cases starting after the age of 50 years, although severe disease can also be encountered in these forms. Variations in penetrance are frequent, particularly with *PAP1*, *PRPF31* and *RP1* genes.

Three genes, *RHO*, *RP1*, and *RDS*, account for approximately 25-30%, 5-10%, and 5-10% of adRP cases, respectively (Berson *et al.* 2001, Sohcki *et al.* 2001). More than 100 *RHO* mutations have been reported but one, P23H, with distinct sectorial disease, is found in approximately 10% of American patients with adRP. *RDS* mutations are associated with clinical phenotypes ranging from RP to macular degeneration to complex maculopathies. Of the *RP1* mutations known, Arg677stop and 2280del5, account for half of adRP cases caused by this gene. Other cloned adRP genes, such as *PRPF31*, cause a substantial fraction of cases, but the specific prevalences are not yet known. Table-13 summarizes the list of genes causing autosomal dominant RP (adRP) in chromosomal order.

Autosomal Recessive RP

Autosomal recessive RP accounts for 5-20% of cases. Autosomal recessive forms start typically during the first decade, although some mild forms can be encountered. Most of the arRP genes are rare, causing 1% or fewer cases, but *RPE65* (expressed in the RPE), and *PDE6A* and *PDE6B* (phosphodiesterase subunits in the phototransduction cascade), cause 2-5% of cases; mutations in *USH2A*, which may also cause Usher syndrome, may account for up to 5% of arRP cases. Mutations in a few genes are common causes of arRP in specific populations, such as *RP25* in Spain, but rare elsewhere. The symptoms of these diseases may overlap with other autosomal recessive retinopathies. In particular, autosomal recessive, early-onset RP and Leber congenital amaurosis (LCA) are very similar. Table-14 summarizes the list of genes causing autosomal recessive RP (arRP) in chromosomal order.

X-Linked RP

X-linked RP accounts for 5-15% cases. X-linked forms also start early and are frequently associated with myopia. Although transmission is recessive in most cases, there are some families in which dominant inheritance with affected females is found.

RPGR (also called *RP3*) and *RP2* are the most common causes of XlRP. Linkage studies suggest that they account for 70-90 % and 10-20 %, respectively, of X-linked RP. Earlier studies of *RPGR* failed to find mutations in a majority of families mapped to this locus,

Table-13: Genes Causing Autosomal Dominant RP (adRP) in Chromosomal order.

Locus Name	Gene	Locus	Product	Also Causes	Percent of adRP
RP18	*HPRP3*	1q21.2	Pre-mRNA splicing factor 3		Several families
RP4	*RHO*	3q22.1	Rhodopsin	Recessive RP; dominant CSNB	25-30%
RP7	*RDS*	6p21.2	Peripherin 2	Dominant MD; digenic RP with *ROM1*; dominant adult vitelliform MD	5-10%
RP9	*PIMIK*	7p14.3	Pim-1 kinase		Unknown
RP10	*IMPDH1*	7q32.1	Inosine monophosphate dehydrogenase 1		3-5%
RP1	*RP1*	8q12.1	Oxygen-regulated protein 1 (RP1 protein)		5-10%
	ROM1	11q12.3	Retinal outer segment membrane protein 1	Digenic RP with *RDS*	Rare
RP27	*NRL*	14q11.2	Neural retina-specific leucine zipper protein		Rare
RP13	*PRPF8*	17p13.3	Pre-mRNA splicing factor C8		Unknown
RP17		17q22	Unknown		Unknown
	FSCN2	17q25	Retinal fascin homolog 2, actin bundling protein		3% of Japanese with adRP
CORD2	*CRX*	19q13.3	Cone-rod homeobox protein	dCORD, dominant and recessive LCA	Rare
RP11	*PRPF31*	19q13.4	Pre-mRNA splicing factor 31		15-20%

Table-14: Genes Causing Autosomal Recessive RP (arRP) in Chromosomal order.

Locus Name	Gene	Locus	Product	Also Causes	Percent of arRP
LCA2, RP20	*RPE65*	1p31.2	Retinal pigment epithelium-specific 61 kDA protein	LCA (7-16%)	2%
RP19	*ABCA4*	1p22.1	Retinal-specific ATP-binding cassette transporter, retinal	Recessive Stargardt disease, and cone-rod dystrophy	Rare
RP12	*CRB1*	1q31.3	Crumbs protein homolog 1	Recessive RP with para-arteriolar preservation of the RPE (PPRPE); LCA (9-13%)	Rare
	USH2A	1q41	Usherin	Usher syndrome, type 2	4-5%
RP28		2p16-p11	Unknown		One family
	MERTK	2q13	C-mer protoonco gene receptor tyrosine kinase		Rare
RP26		2q31-q33	Unknown		Rare
	SAG	2q37.1	S-arrestin	Recessive Oguchi disease	Rare
	RHO	3q22.1	Rhodopsin	Dominant RP; Dominant CSNB	Rare
CSNB3	*PDE6B*	4p16.3	Rod cGMP-specific 3', 5'-cyclic phosphodiesterase beta-subunit	Dominant CSNB	3-4%
	CNGA1	4p12	cGMP-gated cation channel alpha 1 subunit		Rare
RP29		4q32-q34	Unknown		Rare; 4 families
	LRAT	4q32.1	Lecithin reitnol		Unknown

			acyltransferase		
	PDE6A	5q33.1	Rod cGMP-specific 3', 5'-cyclic phosphodiesterase alpha-subunit		3-4%
RP14	*TULP1*	6p21.3	Tubby-related protein 1		Rare
RP25		6q14-q21	Unknown		10-20% of arRP in Spain
	RGR	10q23.1	RPE-retinal G protein-coupled receptor	Dominant choroidal sclerosis	Unknown
	NR2E3	15q23	Nuclear receptor subfamily 2 group E3	Recessive enhanced S-cone syndrome	Rare; found in Sephardic Jews in Portugal
	RLBP1	15q26.1	Cellular retinaldehyde-binding protein	Recessive Bothnia dystrophy; recessive retinitis punctata albescans; recessive Newfoundland rod-cone dystrophy	Unknown
RP22		16p12.3-p12.1	Unknown		Rare
	CNGB1	16q13	Rod cGMP-gated channel beta subunit		

Adapted from RetNet (http://www.sph.uth.tmc.edu/RetNet/)

however, recent identification of an additional exon in *RPGR* (ORF15) has been substantially increased the mutation detection rate (Bader *et al.* 2003).

ORF15 is also the site of most or all dominant-acting mutations at this locus (Rozet *et al.* 2002, Bader *et al.* 2003). In general, the multiplicity of XlRP genes in close proximity to each other makes gene mapping and mutation detection difficult.

An important diagnostic complication is that carrier females may express mild retinal degeneration (Souied *et al.* 1997, Grover *et al.* 2000). Therefore, families with X-linked inheritance of RP with affected females can be mistaken for families with adRP. Typically, though, retinal disease in affected females with XlRP is much less severe than that seen in males, in contrast to adRP in which males and females are, on average, equally affected. Table-15 summarizes the list of genes causing X-Linked RP (xlRP) in chromosomal order.

Table 15: Genes Causing X-Linked RP (xlRP) in Chromosomal order.

Locus Name	Gene	Locus	Product	Also Causes	Percent of XlRP
RP23		Xp22			Unknown
RP6		Xp21.3-p21.2	Unknown		Unknown
RP3	*RPGR*	Xp21.1	Retinitis pigmentosa GTPase regulator	Cone dystrophy	70%
RP2	*RP2*	Xp11.2	XRP2 protein		8%
RP24		Xq26-q27	Unknown		Unknown

Adapted from RetNet (http://www.sph.uth.tmc.edu/RetNet/)

Digenic RP

Rare cases have been described in which heterozygous mutations in *ROM1* in combination with heterozygous mutations in *RDS* cause digenic RP. These forms are inherited in a pseudo-dominant pattern.

Digenic RP is caused by the simultaneous presence of a mutation in the *RDS* gene and a mutation in the *ROM1* gene (Dryja *et al.* 1997). In all cases reported, the same *RDS* mutation (L185P) was found, although three different *ROM1* mutations were identified in these families.

Mitochondrial Genes Causing Non-Syndromic RP

Some Mitochondrial genes have been identified causing Non-Syndromic RP (Table-16).

Table 16: Mitochondrial Genes Causing Non-Syndromic RP.

Gene	Locus	Product	Percent RP
MTTS2	MtDNA	Mitochondrial sevine tRNA2	Rare

Isolated (simplex) cases:

Isolated (simplex) cases represent 10-40% of all RP patients and may be the result of a new autosomal dominant or X-linked mutation or autosomal recessive inheritance, or they may be individuals with relatives who are affected (perhaps mildly) but whose disease is not known to the patient by mode of inheritance.

2. Syndromic retinitis pigmentosa

Many syndromes associated with various types of pigmentary retinopathies, including RP. These syndromes are summarized below.

- ***Usher syndrome***:

Usher syndrome is the most frequent syndromic form in which typical RP is associated with neurosensory deafness. About 14 % of RP cases are Usher syndromes (Boughman *et al.* 1983). Deafness, generally congenital and stable, may be profound (type 1) or moderate/medium (type 2). In some cases (type 3), deafness occurs during the first decade and worsens progressively. At least 12 genes are responsible for Usher syndrome (Ahmed *et al.* 2003).

- ***Bardet Biedl syndrome :***

Bardet Biedl syndrome is less frequent with a prevalence of 1/150.000 (Beales *et al.* 1999) but the phenotype is characteristic. It associates RP (often of the cone-rod dystrophy type) with obesity, mental retardation, post axial polydactyly, hypogenitalism and renal abnormalities that lead to renal failure. At least 7 genes are responsible, with cases of triallelic digenic inheritance.

RP is not a curable disorder. Research, at molecular level is in progress. Genes responsible for this disorder have been reported from different populations. Pakistani population is an interesting one because of close relative marriage and particularly in first cousin marriages, which are preferred here. Because of inbreeding in human population it is expected that recessive form of disorder shall appear. The aim of the present study is to search for any new locus for this disorder and how consanguinity influences in the appearance of this disorder. These contributions in this regard may be helpful in future research regarding gene therapy of this disorder.

RESULTS

Epidemiology

A Pakistani consanguineous kindred suffering from night blindness was identified (Fig.24). The surname of the family is Arian and they are settled in the District Mianwali. The kindred were very poor and non-educated. The members of the family were very cooperative in providing information they were asked. Mostly consanguineous marriages contracted in the family resulted in a higher number of affected children.

The kindred spans on five generations and consists of 45 individuals, out of which twenty seven were live and eighteen were dead at the time of data collection. Of the live members, nine were affected including six males and three females.

Individual V-16 is the proband that was first diagnosed for the disease and with his help the disorder was traced back in the family.

In the first generation, first cousin marriage between normal man (I-1) to a normal woman (I-2) produced one normal son (II-2) and one affected son (II-3). Un-related normal man (I-3) married to normal woman (I-4) and had one normal daughter (II-4) and one normal son (II-5).

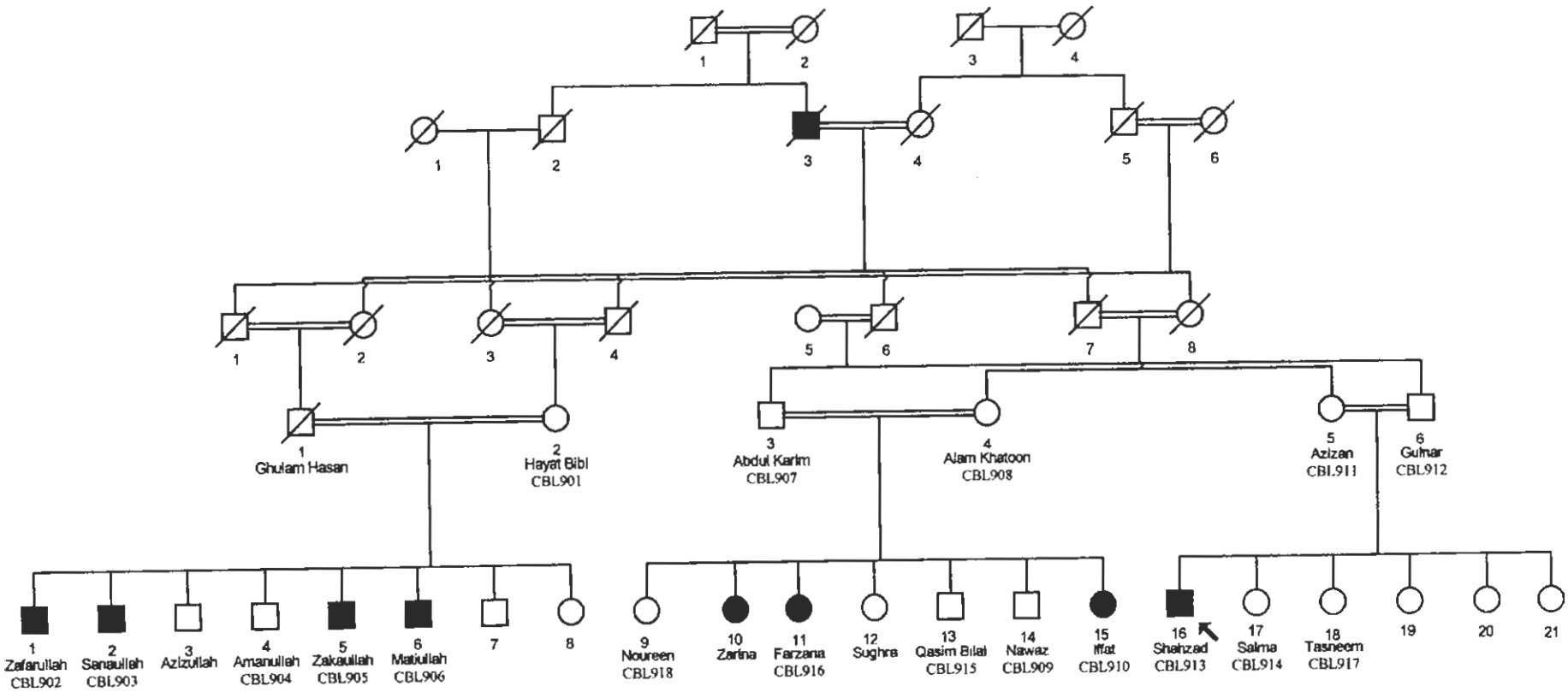


Fig.24 Pedigree of a consanguineous Pakistani family suffering from autosomal recessive retinitis pigmentosa sine pigmento, 9CBL.

In the second generation un-related normal woman (II-1) was married to normal man (II-2) and produced one normal daughter (III-3). Affected man (II-3) was married to his normal first cousin (II-4) and produced one normal daughter (III-2) and three normal sons (III-4, III-6 and III-7). A first cousin marriage between normal man (II-5) and woman (II-6) produced one normal son (III-1) and one normal daughter (III-8).

In the third generation first cousin marriage between normal man (III-1) and woman (III-2) produced one normal son (IV-1). Another first cousin marriage between (III-3) and (III-4), both normal for this disorder, had one normal daughter (IV-2). Other first cousin marriages were contracted between III-5 and III-6 and between III-7 and III-8.These were normal for this disorder. They produced two normal sons (IV-3 and IV-6) and two daughters (IV-4 and IV-5), respectively.

In the fourth generation three cousin marriages were contracted who were normal for this disorder. First was between (IV-1) and (IV-2) who have eight children. Their four sons (V-1, V-2, V-5 and V-6 were affected. Other sons V-3, V-4 and V-7 and a daughter (V-8) were normal. Second was between (IV-3) and (IV-4) who have seven children. Three of their daughters (V-10, V-11 and V-15) were affected, and two daughters (V-9; V-12) and two sons (V-13 and V-14) were normal. The third marriage was between (IV-5) and (IV-6). They have five normal daughters (V-17, V-18, V-19, V-20 and V-21) and one affected son (V-16, proband).

The pedigree shows an autosomal recessive mode of inheritance. The affected members were diagnosed for Retinitis Pigmentosa Sine Pigmentosa.

Clinical assessment:

Two affected members (V-15 and V-16) of the family 9CBL were clinically examined. Their ages were 17 years and 08 years respectively.

Age of onset:

All the affected members of the family had congenitally night blindness.

Clinical description:

Clinical description of the affected individuals is summarized in the table 17.

Table-17: Clinical description of the affected individuals in the family 9CBL.

Patient number	Visual Acuity		Stippled macula	Pale optic disc	Bony spicules	Attenuated blood vissels
	Right	Left				
V-15	6/24	6/18	+	+	-	+
V-16	6/18	6/18	+	+	-	+

Ophthalmologist observed visual acuity 6/24, 6/18 and 6/18, 6/18 in right and left eye of V-15 and V-16, respectively. And no improvement was seen with glasses. Retina on fundus examination of both affected individuals showed stippled maculae, attenuated blood vessels, pale optic disc and no bony spicules, pathognomonic of retinitis pigmentosa.

Parents observations:

Individuals with ophthalmological disorder had reduced vision at night, since birth. They also noted lack of visual responses at night and in dim light in affected children.

Associated abnormalities:

Any associated abnormality like polydactly, obesity, hypogonadism, hearing loss, mental retardation, epilepsy, neurological disorders etc. was not found among them. They were normal physically and mentally except ocular disorder.

Proposed diagnosis:

The proposed diagnosis made by the clinician is Retinitis Pigmentosa Sine Pigmento.

Molecular Studies:

The members of this family were diagnosed for Retinitis pigmentosa sine pigmento, the mode of inheritance ascertained for this disease was autosomal recessive (Fig.24). Nineteen family members were processed for molecular study. Of these 12 members were normal and seven were affected.

Since the affected individuals were congenitally night blind, therefore, initially the affected members and their close degree relatives were screened to exclude the linkage to the regions of the previously described genes involved in LCA on chromosomes 1,6,14,17 and 19 (Table 8; pp 43-44). During exclusion analysis, all the

known loci for LCA were excluded. Then the known loci of autosomal recessive Retinitis Pigmentosa (arRP) (Table-18) were also excluded. Table-19 lists the exclusion of the known loci of autosomal recessive and autosomal dominant Cone-Rod dystrophies. Then the samples were analyzed for the known loci of autosomal dominant RP (Table-20). It was observed that the samples were linked at $8q^{11}$-q^{13} with microsatellite markers, D8S285 and D8S1113 (Fig.25). These markers showed linkage for autosomal dominant inheritance (Xu *et al.*1996; Jacobson *et al.*2000)

Two-point linkage analysis resulted in lod score of 3.11 for marker D8S285 at recombination fraction 0. Flanking marker D8S1815 was spaced at 3.73 cM and D8S1113 was spaced at 6.89 cM. Marker D8S1815 yielded a lod score of 2.74 at recombination fraction 0. Lod score of -infinity was obtained for D8S1113 on the distal side of marker D8S285.At marker D8S285 lod score of 3.03 was obtained at recombination fraction 0.01 and 2.71 at recombination fraction 0.05. The analysis also resulted, a lod score of 1.95 for marker D8S1722 at recombination fraction 0 (Table-21).

The affected haplotype was represented by 1,1,1,3,1(Fig.26). Normal members IV-1, IV-2, IV-3, IV-4, IV-5, V-4, V-9,V-13,V-17 and V-18 are carrier for the disease with the exception of V-14 and genotype of IV-6 is difficult to explain due to the non amplification of markers. The affected members V-11 and V-15 are homozygous for all the five markers. Affected members V-1, V-2 and V-5 are heterozygous for marker D8S1477.They inherited affected maternal haplotype and partly affected haplotype from father. Affected member V-6 has inherited affected paternal haplotype while maternal haplotype shows recombination between markers D8S285 and D8S1113.This affected member (V-6) hence is hetrozygous for markers D8S1477 as well as for D8S1113.In affected member V-16 recombination in maternal haplotypes markers D8S1477 and D8S1722 and another exchange is between markers D8S285 and D8S1113. Its (V-16) paternal haplotype is homozygous for this disease-region between markers D8S1815 and D8S285.The recombination event is not clear because in paternal haplotypes at markers D8S1815 and D8S285 amplification did not take place.

Table 18: Known loci, genes and markers of autosomal recessive Retinitis Pigmentosa (RP) as described by other authors.

Sr. No.	Gene	Chromosomal Location	Clinical Classification	Mode of Inheritance	Markers	Reference	This Study
1	RP19 (ABCR)	$1p^{21}$-p^{22}	RP	AR	D1S1665, D1S2841	Allikmets, 2000	Excluded
2	RP12/PPRPE	$1q^{31}$-$q^{32.1}$	RP	AR	D1S1660, D1S1678	van Soest *et al.*, 1994	Excluded
3	USH2A	$1q^{41}$	RP	AR	GATA124FO8, D1S2141	Bhattacharya *et al*,.2002 Eudy *et al.* 1998	Excluded
4	RP28	$2p^{11}$-p^{16}	RP	AR	D2S1394, D2S286	Gu *et al.*,1999	Excluded
5	RP26	$2q^{31}$-^{33}	RP	AR	D2S1391, D2S364	Bayes *et al.*, 1998	Excluded
6	SAG	$2q^{37.1}$	RP	AR	D2S427, GATA178GO9	Ngo *et al.*, 1990	Excluded
7	RP4(RHO)	$3q^{21}$-q^{24}	RP	AR	D3S3637, D3S1764	Dryja, 1991	Excluded
8	PDDEB	$4p^{16.3}$	RP	AR	D4S2366, D4S2935	Bateman *et al.*, 1992	Excluded
9	CNGA1	$4p^{12\text{-cen}}$	RP	AR	D4S174, D4S1627, D4S401	Dryja, 1995	Excluded
10	RP29	$4q^{31}$-q^{34}	RP	AR	D4S2368, D4S2431	Hameed *et al.*, 2001	Excluded
11	LRAT	$4q^{31.2}$	RP	AR	D4S3028, D4S3035, D4S303, D4S2417	Ruiz *et al.* 2001	Excluded
12	PDE6A	$5q^{31.2}$-q^{34}	RP	AR	D5S816, D5S1480,D5S820	Pittler *et al.*, 1990	Excluded
13	TULP1,RP14	$6p^{21.3}$	RP	AR	D6S291, D6S1019	Knowles *et al.*, 1994	Excluded
14	RP25	6ncen-q^{15}	RP	AR	D6S257, D6S1053	Ruiz *et al.*, 1998	Excluded
15	RGR	$10q^{23}$	RP	AR		Chen *et al.*, 1996	Excluded

Table 19: Known loci, genes and markers of autosomal recessive and dominant Cone-Rod Dystrophy (CORD) as described by other authors.

Sr. No.	Gene	Locus	Disease	Mode of Inheritance	Markers	Reference	This Study
1	ABCR	$1p^{21}$-p^{22}	CORD	AR	D1S1665, D1S2841	Nasonkin *et al.* 1998	Excluded
2	GUCAIA	$6p^{21.1}$	CORD	AD	GGAA15B08, D6S1017	Payne *et al.* 1998	Excluded
3	CORD9	$8p^{12}$-q^{11}	CORD	AR	D8S1477, D8S1110	Danciger *et al.* 2001	Excluded

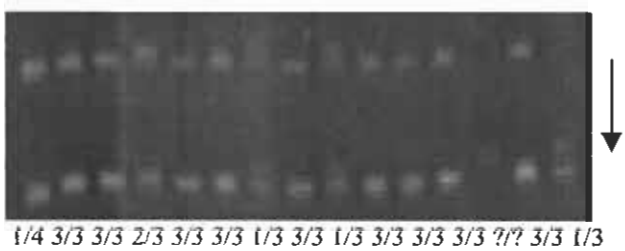
Table 20: Known loci, genes and markers of autosomal dominant Retinitis Pigmentosa as described by other authors.

Sr. No.	Gene	Locus	Disease	Mode of Inheritance	Markers	Reference	This Study
1	HPRP3,RP18	$1p^{21.1}$	RP	AD	D1S514	Chakarova *et al.* 2002, Xu *et al.* 1998	Excluded
2	RDS, RP7	$6p^{21.2\text{-}cen}$	RP	AD	D6S1959, D6S2439	Ali *et al.* 2000	Excluded
3	RP9, PIMIK	$7p^{15.1}$-p^{13}	RP	AD	D7S1808, D7S817	Keen *et al* 2002	Excluded
4	RP1	$8q^{11}$-q^{13}	RP	AD	D8S285, D8S1113	Xu *et al.* 1996, Jacobson *et al.* 2000	Shown linkage
5	RP1	$8q^{11}$-q^{13}	RP	AR	D8S1815,D8S285, D8S1113	This study	Linked

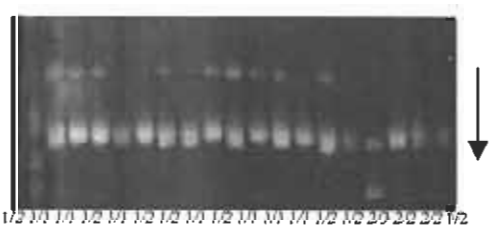
D8S1477

D8S1722

D8S1815



D8S285



D8S1113

Fig.25. Gel photograph of markers, D8S1477, D8S1722, D8S1815, D8S285 and D8S1113 used for adRP, in the family 9CBL.

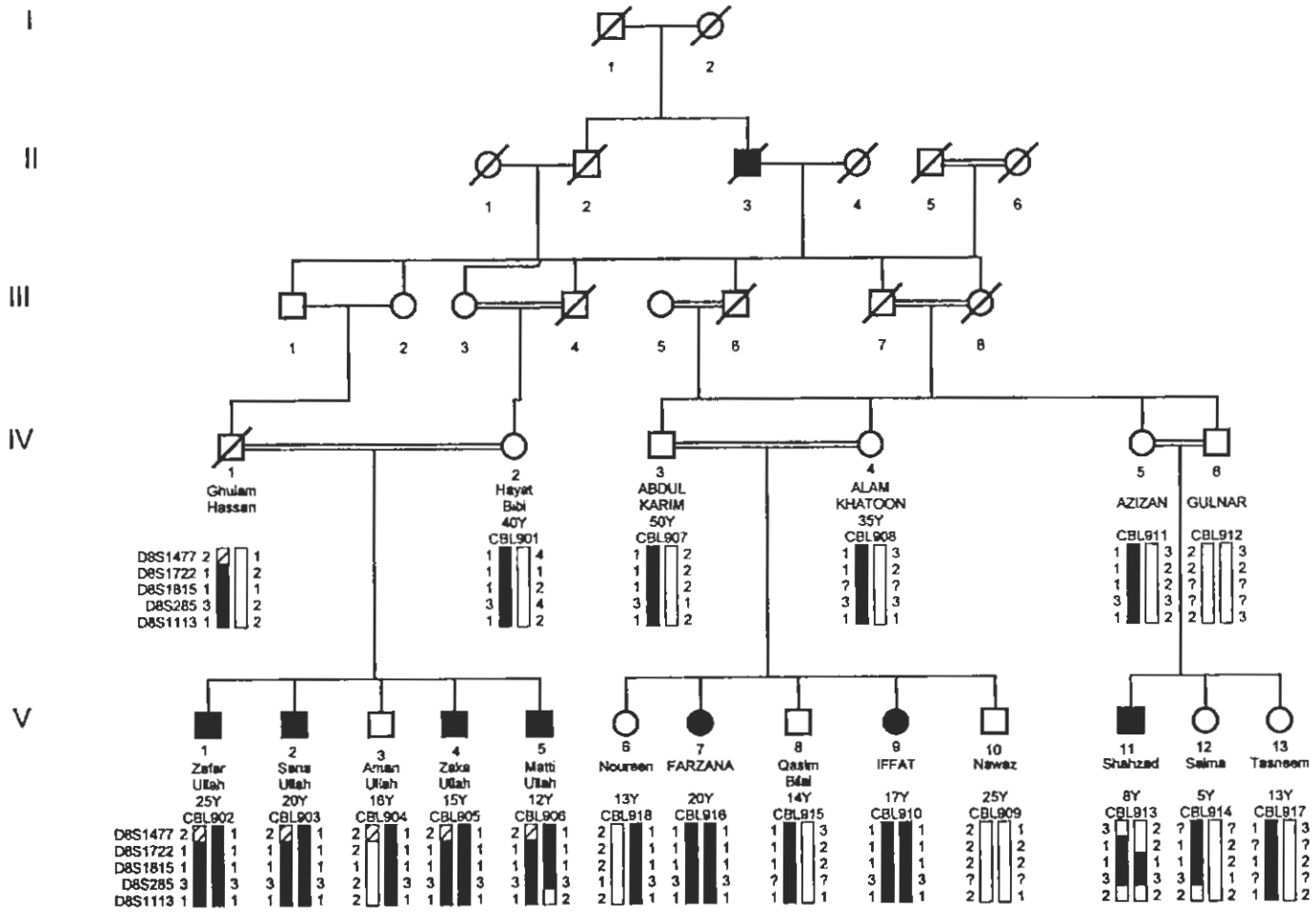


Fig.25. Part of pedigree 9CBL (Fig.24), showing homozygosity of the disease haplotype in affected individuals. Shaded bars denote the disease haplotype and cross lines represent the cross overs. Haplotype of IV-1 is assumed from his progeny.

Table-21:Two point lod scores and the genetic distances between the microsatellite markers analysed in the family 9CBL.

Marker	Marshfield map position (cM)	LOD scores at θ								
		0.0	0.01	0.05	0.1	0.2	0.3	0.4	Zmax	θ max
D8S1477	60.34	0.19	0.32	0.66	0.80	0.71	0.42	0.13	0.80	0.1
D8S1722	62.47	1.95	1.92	1.77	1.54	1.03	0.54	0.15	1.95	0.0
D8S1815	67.27	2.74	2.66	2.35	1.96	1.21	0.57	0.15	2.74	0.0
D8S285	71.00	3.11	3.03	2.71	2.33	1.59	0.87	0.28	3.11	0.0
D8S1113	77.89	-∞	-2.82	-0.92	-0.27	0.10	0.12	0.05	0.12	0.3

Discussion

Retinitis pigmentosa (RP) is genetically heterogeneous disease which causes hereditary defects in vision leading to blindness (Boughman *et al.* 1980; Kaplan *et al.* 1990). Mode of inheritance of RP is in three mendelian forms autosomal dominant, autosomal recessive and X-linked but sporadic or isolated cases of RP also occur (Greenberg *et al.* 1993). The most frequent form of inherited retinopathy is retinitis pigmentosa (RP) with an approximate incidence of 1 in 3,500 individuals worldwide (sullivian *et al.*1999).

RP is inherted most frequently as autosomal recessive trait. Fifteen different loci have been mapped on chromosome 1p21-22 (Allikmets, 2000),1q31-32.1(Van Soest *et al.*1994),1q41(Bhattacharya *et al.*,2002; Eudy *et al.*1998),2p11-16 (Gu *et al.*1999),2q31-33 (Bayes *et al.*1998), 2q37.1 (Ngo *et al.*1990), 3q21-24 (Dryja *et al.*1991), 4p16.3 (Bateman *et al.*1992), 4p12-cen (Dryja *et al.*1995), 4q31-34 (Hameed *et al.*2001), 4q31.2 (Ruiz *et al.*2001), 5q31.2-34 (Pittler *et al.*1990), 6p21.3 (Knowles *et al.*1994), 6cen-q15 (Ruiz *et al.*1998) and 10q23 (Chen *et al.*1996). Gu *et al.* 1999 have investigated in an Indian family, with multiple consanguineous marriages, that close linkage exist between the disease locus and D2S380, D2S441, D2S291, and D2S1394 with maximum lod scores between 1.51-3.07 at θ=0.00. They found that analysis of multiply informative meioses maps the locus (RP28) for ArRP in this family between D2S1337 and D2S286 on 2p11-15.

Finckh *et al.* (1998) collected DNA samples in the Indian subcontinent from a large consanguineous ArRP families. They indicated that linkage data mapped the diseased locus (RP22) in an approximately 16cM region between D16S287 and D16S420 on the proximal short arm of chromosome 16.They did not find mutation by direct sequencing in the gene(CRYM) encoding μ crystallin, which maps in this critical region.

In a large Pakistani family with several consangineous marriages in which autosomal recessive retinitis pigmentosa is segregating, close linkage between the disease locus and six loci on chromosome 1q (D1S158, F13B, D1S422, D1S412, D1S413 and D1S53) with maximum lod scores ranging from 0.988-4.657 at θ=0.065-0.235 (Leutelt *et al.*1995).

This study is based on a family with many consangineous marriages inheriting RP in an autosomal recessive form. Close linkage exists between disease locus and D8S1477, D8S1722, D8S1815, D8S285 and D8S1113.Two point analysis resulted in lod score of 3.11 at θ=0.00 with D8S285. Makers D8S1815 and D8S285 are 3.73cM apart. The linkage data mapped the disease locus (RP1) for ArRP between D8S1815and D8S285 on 8q11-13.

Genetic mapping of RP has also been done in other populations involving families where they show autosomal dominant inheritance of retinitis pigmentosa. Xu *et al.* (1996) mapped one form of autosomal dominant retinitis pigmentosa (adRP) on chromosome 8q11-q22 between D8S589 and D8S285, which are about 8 cM apart, by linkage analysis in an extended family ascertained from USA. They studied a multigeneration Australian family with adRP and found close linkage without recombination between the disease locus and D8S591, D8S566, and D8S166 (Zmax = 1.137-4.650 at theta = 0.00), all mapped in the region known to harbor RP1. They assumed that the mutation of the same gene is responsible for the disease in both families, the analysis of multiply informative meioses in the American and Australian families places the adRP locus between D8S601 and D8S285, which reduces the critical region to about 4 cM.

Pierce *et al.* (1999) mapped autosomal dominant retinitis pigmentosa (RP) locus, through linkage studies to a 4-cM interval at 8q11-13. They described a new photoreceptor-specific gene that maps in this interval. This gene consists of at least 4 exons that encode a predicted protein of 2,156 amino acids. A nonsense mutation at codon 677 of this gene is present in approximately 3% of cases of dominant RP in North America. The authors also detected two deletion mutations that cause frameshifts in three other families with dominant RP. They suggest that mutations in this gene cause dominant RP, and that the encoded protein has an important but unknown role in photoreceptor biology.

Sullivan *et al.* (1999) have identified two mutations in a novel retina-specific gene from chromosome 8q that cause the RP1 form of autosomal dominant RP in three unrelated families. The protein encoded by this gene is 2,156 amino acids and its function is not known. They found that two families have a nonsense mutation in codon 677 of this gene (Arg677stop), whereas the third family has a nonsense mutation in codon 679

(Gln679stop). They observed in one family, two individuals homozygous for the mutant gene have more severe retinal disease compared with heterozygous.

Blanton *et al.*(1991) have mapped autosomal dominant retinitis pigmentosa (AdRP) demonstrating linkage between the disease locus (RP1) and DNA markers on the short arm of human chromosome 8. Multipoint linkage analysis, using a simplified pedigree structure for the family (which contains 192 individuals and two inbreeding loops), gave a maximum lod score of 12.2 for RP1 at a distance 8.1 cM proximal to PLAT(plasminigen activator, tissue) in the pericentric region of the chromosome. They pointed out that several families with AdRP with tight linkage to the rhodopsin locus at 3q21-q24 were reported. They are of the view that mutations at two different loci, at least, have been shown to cause AdRP. There is no remarkable clinical disparity in the expression of disease caused by these different loci.

Bowne *et al.* (1999) identified the gene responsible for the RP1 form of autosomal dominant retinitis pigmentosa (adRP) at 8q11-12. The RP1 gene is an unusually large protein, 2156 amino acids in length, but is comprised of four exons only. They are of the view that mutations in RP1 appear to cause at least of 7% of adRP, and also that mutation in other regions of RP1 can cause forms of retinal diseases other than AdRP and whether the background variation in either the mutated or wild-type RP1 allele plays a role in the disease phenotype.

Disease locus (RP1) and segregating markers for autosomal dominant inheritance have been mapped on long arm of chromosome 8 by the above authors. Mapping of locus for autosomal recessive RP has been observed on chromosome 1,2,3,4,5,6 and 10. Pierce *et al.*(1999) have mapped AdRP on 8q11-q13. This is the locus (8q11-q13) where locus for ArRP has been mapped. It is suggested that mutation at this locus has taken place resulting in recessive form of RP.

GENERAL DISCUSSION

General Discussion

This study includes epidemiological and molecular studies of five consanguineous Pakistani families afflicted with different types of congenital blindness. These families are diagnosed for Leber's congenital amaurosis (LCA), macular degeneration, retinitis pigmentosa sine pigmento and unusual phenotype of fundus dystrophy. All these families are highly inbred and it is seen that there are 60% consanguineous marriages in these families.

Consanguinity in Pakistan (Punjab) population has been reported as 48% by Shami *et al.* (1989). The effects of consanguinity on the frequency of RP patients in Japanese population showed that decline in consanguineous marriages revealed a relative decline in the frequency of autosomal recessive cases (Hayakawa *et al.* 1997). As a consequence of consanguineous marrriages increase in autosomal recessive RP was also reported in Turkish population (Atmaca *et al.* 1995). Autosomal recessive cases revealed consanguinity as the main characteristic (49 out of 53) in the Indian population studies which resulted in an early onset and severe progression of disease (Vinchurkar *et al.* 1996). A strong influence of parental consanguinity on the inheritance of ocular disorders is observed in this study.

In certain parts of Asia and Africa, consanguineous marriage constitutes 20% to 55% of all marriages and it is a social practice (Bittles *et al.* 1991; www.consang.net). Consanguinity is widely practiced by Muslims all over India and neighbouring countries like Pakistan, Bangladesh and Sri Lanka. This is comparable to present findings. Consanguinity and health, an intensely debated and controversial issue in human genetics was investigated in the United States of America as early as in 1858 by Bemiss. Several studies show collectively that despite homozygosity by descent, there is no clear evidence to prove that complex traits like lowered intellectual capacity, spontaneous abortions and stillbirths are influenced by consanguinity (Bittles *et al.* 1991). However it is well known that consanguinity and autosomal recessive diseases are closely associated. In 1902 Garrod studied the association between autosomal recessive biochemical disorder and consanguinity. Present study is also comparable with above findings which include all autosomal recessive families afflicted with congenital blindness.

In North America and Western Europe, the incidence of consanguineous marriage at the first cousin level is approximately 0.5% (Bittles *et al.* 1991). The trends in consanguineous marriage practice in developed countries have declined in the 20th century but not so in the west and south Asian regions (Bittles and Hussain, 2000). Over a period of four decades consanguineous marriage practices have not changed in Pakistan, similarly no change was observed in the Muslim communities in India (Bittles and Hussain, 2000). Our epidemiological survey is also comparable with the above findings.

Non-biological factors like economic status and levels of education were analyzed to see its effect on the development of ocular diseases. A higher number of patients were the offspring of low paid government servants or farmers and having attained only school level education or non-educated (Mehtab,2000).

Congenital ocular disorders like lebers congenital amaurosis have been mapped on 17p13.1(Camuzat *et al.*1995; Camuzat *et al.*1996; perrault *et al.* 1996), 1p31.2(Gu *et al.* 1997; Marlhens *et al.* 1997; Morimura *et al.* 1998); 14q24 (Stockton *et al.*1998); 17p13.2 (Hameed *et al.*2000; Sohocki *et al.* 2000b); 6q11-q16 (Dharmaraj *et al.*2000; Mohamed *et al.* 2003); 14q11.2 (Dryja *et al.* 2001; Cremers *et al.*2002); 19q13.32(Freund *et al.*1998; Jacobson *et al. 2000*; Swaroop *et al.*1999; Rivolta *et al.*2001; Perrault *et al.* 2003); 1q31.3 (den Hollander *et al.* 2001; Lotery *et al.* 2001) and 1p36 (Keen *et al.* 2003).

Present study mapped the disease locus for 3CBL family of Leber's congenital amaurosis at $17p^{13.1}$ by linkage analysis. At this locus two candidate genes (AIPL1 and GUCY2D) are present. Involvement of AIPL1 is possible as Sohocki *et al.* (2000b) analyzed that D17S796 and D17S1881 are tightly linked flanking markers of AIPL1 and our study also gave a maximum lod score of 4.75 at $\theta = 0$ with D17S796. Several mutations in the LCA4 and adCORD cases have been reported in different exons of the AIPL1 gene (Sohocki *et al.*2000 a,b; Damji *et al.* 2001).They also suggested that of these Trp278x mutation appears to be very common and has been reported in several families from different ethnic backgrounds around the world, including six families of Pakistani origin. The presence of a novel Thr39Asn mutation in this gene in yet another Pakistani family suggests a high rate of AIPL1 gene mutations in Pakistani families (Khaliq *et al.*

2003). Many LCA families of Pakistani origin mapped on $17p^{13}$ have been shown to have AIPL1 gene mutations. The interesting observation is that none of the $17p^{13.1}$ linked Pakistani families that had been reported so far, had disease associated mutations in the GUCY2D gene (Khaliq *et al.* 2003). It is suggested that AIPL1 is the candidate gene which is involved in the pathogenesis of autosomal recessive Leber 's congenital amaurosis.

In this study a Pakistani consanguineous family 7CBL suffering from autosomal recessive macular degeneration, linkage studies revealed the presence of disease locus at 6p21.2 where a candidate gene peripherin/RDS for eye diseases is present, which is involved in dominant RP, dominant macular degeneration, digenic RP with ROM1 and adult vitelliform macular degeneration (Ali *et al.*2000; Dryja *et al.*1997; Felbor *et al.*1997; Kajiwara *et al.* 1994). Two point lod score gave maximum value of 3.29 at recombination fraction 0.0 for microsatellite marker D6S1019 and lod score of 3.0 at recombination fraction 0.05 for microsatellite marker D6S1017 which is a tightly linked marker of RDS gene.

Present study mapped the locus for autosomal recessive macular degeneration at 6p21.2, which was a previously reported locus for autosomal dominant macular degeneration from non-Asian populations (Farrar *et al.* 1991; Kajiwara *et al.* 1991). It is suggested that mutation at this locus might have taken place resulting in recessive form of macular degeneration or this is just possible that in this family from Pakistan this disorder may be controlled by recessive gene. These results suggest that inheritance for macular degeneration could be genetically hetrogeneous (Michaelides *et al.* 2003a).

In this study the disease was mapped at locus 22q12.1-q13.2 through homozygosity mapping with microsatellite markerD22S280 and D22S685 for 8CBL family of fundus dystrophy. The disease is recessively controlled in this family. The locus identified in this study is the same as has been reported for dominantly controlled Sorby's fundus dystrophy. The symptoms indicated in clinical examination are similar to that of Sorby's fundus dystrophy with unusual presence of menining bulging with alopecea in occipital region. Present disease seems to be one related to Sorsby's fundus dystrophy but is recessively controlled. It is suggested that in affected individuals TIMP3 gene is involved in the development of this disorder.

In family 9CBL of autosomal recessive RP close linkage exists between disease locus and D8S1477, D8S1722, D8S1815, D8S285 and D8S1113. Two point analysis resulted in lod score of 3.11 at θ=0.00 with D8S285. Makers D8S1815 and D8S285 are 3.73cM apart. The linkage data mapped the disease locus (RP1) for ArRP between D8S1815 and D8S285 on $8q^{11-13}$. Mapping of locus for autosomal recessive RP has been observed on chromosome 1,2,3,4,5,6 and 10. Pierce *et al.* (1999) have mapped AdRP on 8q11-q13. This is the locus (8q11-q13) where locus for ArRP has been mapped in this study. It is suggested that mutation at this locus has taken place resulting in recessive form of RP. This study suggests the genetic hetrogenity of autosomal recessive retinitis pigmentosa sine pigmento.

This study suggests that consanguineous marriages have a profound effect on the development of recessive eye disorders, and due to homozygosity autosomal recessive inheritance pattern is exhibited in all the families. A high percentage (60%) of consanguineous marriage has been observed in all the families studied here.

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