ANALYSIS AND MAPPING OF GENETIC
DISORDERS IN PAKISTAN

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By

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SUMMARY:

This study was carried out to localize/identify the loci and genes responsible for causing retinal dystrophies in the Pakistani population. For this, a number of large inbred families as well as a large number of patients suffering from Leber congenital amaurosis (LCA), cone-rod dystrophy (CRD) and retinitis pigmentosa (RP) were collected with informed consent and screened for disease causing mutations.

The families were initially screened for all the known retinal dystrophy loci. Following the exclusion of all the known loci, a genome wide search was carried out using polymorphic microsatellite markers. For gene identification, databases were searched for candidate genes within the critical disease interval. For mutation detection in the retinal dystrophy panel, single stranded conformational polymorphism (SSCP) and denaturing high performance liquid chromatography (DHPLC) were carried out. Direct sequencing was performed to find out the disease causing mutation in the genes. The results are as follows.

During screening for the known retinal dystrophy loci, three families (3330RP, 111RP and 010LCA) were found to be linked at the CRB1 gene locus on chromosome 1q31-32.1. Among these, two families were suffering from arRP with para-arteriolar preservation of retinal pigment epithelium (PPRPE) and one family with LCA. Sequencing analysis of the CRB1 gene revealed three novel disease-causing mutations. In the 3330RP family a homozygous G to A substitution was found at codon 846 in exon 7. This mutation replaced glycine
with an arginine residue. Another T to C substitution at codon 1116 in exon 9 changed leucine with a proline in the 111RP family. Family 010LCA had a homozygous T to C substitution at codon 989 that changed isoleucine with a threonine residue.

Another LCA family (011LCA) was found to be linked to chromosome 17p13.1. Mutation screening of the APL1 gene (present on 17p13.1) revealed a homozygous C to A transversion at codon 116 in exon 2. This mutation changed the amino acid threonine with asparagine.

Two other families (1CRD and 4CRD) suffering from CRD were observed to be linked to chromosome 14q11. This region of the chromosome harbors the RPGRIP1 gene. The RPGRIP1 gene has previously been reported to cause LCA. Mutation screening revealed a homozygous G to T point mutation in exon 16 (Arg827leu) in the 1CRD family. Sequencing of the 4CRD family showed a homozygous G to T substitution in exon 13 (Ala547Ser).

This is the first report showing the involvement of RPGRIP1 gene mutation in the pathogenesis of CRD.

Linkage analysis of three arRP families (442RP, 452RP and 336RP) showed significant linkage with markers D8S285 and D8S1815. This locus contains the RP1 gene that has previously been reported to be associated with autosomal dominant RP. Sequencing analysis of the RP1 gene for 442RP and 452RP revealed a homozygous C to T substitution at nucleotide 1118 (Thr373Ile). Another homozygous 4bp insertion (1461-1465insTGAA) was found in exon 4 in the 336RP family. This insertion produced a stop codon (TGA)
immediately after codon 1461, resulting in the synthesis of a truncated protein of 487 amino acids instead of 2156 amino acids.

A panel of 150 patients was also screened for mutations in the RP1 gene. A heterozygous G to A substitution was found in exon 4 in one patient. This mutation replaced alanine with a threonine at codon 699. These results showed the involvement of the RP1 gene in the pathology of autosomal recessive RP.

The Semaphorin (SEMA4A) gene has been reported to cause severe retinal degeneration in a mouse model. To find out the association of human SEMA4A gene with retinal dystrophies, SSCP analysis was carried out using a panel of 190 patients. Sequencing analysis of the variant bands from SSCP gels revealed two compound heterozygous mutations in two patients with RP and two patients with CRD. These mutations were Asp345His and Phe350Cys. Both the mutations were found to segregate together in all the patients. The parents were heterozygous for each of the two mutations. This suggests that in the autosomal recessive mode of inheritance a single mutation does not lead to the disease state.

Another disease causing mutation was identified in three RP and one LCA patient. This heterozygous G to A mutation was found to change the codon for arginine with that of glutamine (Arg713Gln) suggesting autosomal dominant mode of inheritance. This mutation was not found in the 100 normal control subjects. This is the first report of the involvement of the SEMA4A gene in the pathogenesis of retinal dystrophies in humans.
In addition to this, linkage analysis revealed significant linkage of dystrophy loci in the ZDAR/LCA region. Mutations in the CRB1 gene were identified in some families. The CRB1 gene was then sequenced in all family members. A single nucleotide polymorphism (SNP) was found to be associated with the disease. Performance liquid chromatography (DHPLC) mutation screening revealed a mutation in the CRB1 gene, which was confirmed by denaturing high performance liquid chromatography (DHPLC) and SSCP analysis. The functional significance of the mutation was assessed using para-arteriolar preservation of retinal pigment epithelium (PPRPE; RP12) and CRB1.
homozygous codon 3330RP

111LCA 111RP

G to A

G to A

homozygous T to C

homozygous T to C

proline residue

iso-leucine residue

threonine residue

homozygous T to C

homozygous G to A

14q11 14q11

CRD and CRD

CRD

1CRD and 4CRD

RPGRIp1

RPGRIp1

1CRD

Arg827Leu

Ala547Ser

cone rod dystrophy

D8S1815 452RP

D8S285 442RP

heterozygous 4bp

homozygous 4bp

homozygous G to A

heterozygous G to A

insertion (1461-1465insTGAA)

stop codon (TGA)

24bp

semi-arginine

semaphorein4A

severe retinal dystrophy
ارویassoc
genen RP 

genen تھیم heterozygous G to A (Arg713Gln) گئن

دوسری چناکی کا سبب بنتی دو لہور تبادلے (LCA)

کے ایک مرتین سب پائی گئی۔
CHAPTER 1

INTRODUCTION

The revolution of modern genetics started in 1865 with the discovery and formulation of Mendel's 'laws of inheritance'. However, his work went unnoticed until Garrod (1902) found that alkaptonuria, an inherited disorder, followed Mendelian laws. Further support came from the work of Karl Landsteiner (1901) whose work on the inheritance of human blood groups was also interpreted to obey Mendel's laws.

To formulate the laws of inheritance, Mendel painstakingly spent many years to create pure lines of the pea plant that carried one or two clearly observable properties—plants that produced only round or wrinkled seeds or those with either long or short stems. From his meticulously planned experiments, Mendel concluded that a pair of "factors", one of which was inherited from each of the parents, controlled the characters that were being examined.

The chemical nature of the "factor" remained unknown until Frederick Griffith made a landmark discovery in 1928 (Griffith, 1928). He observed that when mice were injected with a mixture of heat killed virulent pneumococci (S type) and a non-pathogenic strain (R type), most of the animals died and surprisingly, the blood cells of the mice had live S type pneumococci. He concluded that a substance from the dead strain transformed the R type to become virulent. Using "purified" deoxyribonucleic acid (DNA) that contained no proteins or ribonucleic acids (RNA), Avery et al (1944) showed that the transforming agent was in fact DNA. Additional support for DNA came from
Hershey and Chase (1952) who infected *E.coli* with bacteriophage T2 in which the coat protein was labelled with $^{35}\text{S}$ and the DNA with $^{32}\text{P}$. They showed that DNA, not the protein, was required for the production of the phage progeny. Finally, the elucidation of the double helical structure of DNA (Watson and Crick, 1953) laid the foundation of the ‘Golden Era’ of modern genetics (biotechnology and genetic engineering) and gene hunting in the 1970’s and 1980’s. Techniques such as the production of recombinant DNA (Cohen *et al*, 1973; Morrow *et al*, 1974), Sanger’s success in sequencing the genome of a bacteriophage (Sanger *et al*, 1977) and the ability to make millions of copies of a specific piece of DNA in a test tube without using the machinery of any living cell by Kary Mullis (Saiki *et al*, 1985) are a few hallmarks of this era.

When Kornberg discovered nucleic acids duplicating enzymes in bacteria (see Kornberg and Baker, 1992), Watson and Crick’s model of the structure of DNA provided an explanation for a number of vitally important questions. The two antiparallel strands of deoxyribonucleotides that strictly obey the hydrogen bonding of A-T and G-C explained the very basis of DNA replication and hence reproduction. Soon after, it was shown that the sequence of a triplet of nucleotides (codon) codes for an amino acid in the biosynthesis of proteins and this established the co-linearity of the gene and the protein that it codes for.

During the second half of the twentieth century, biology and therefore medicine found a universal language— the language of chemistry. This attracted a large number of workers to move into biology from other scientific disciplines. In addition, modern biologists became increasingly at ease to borrow and use techniques from the physical and chemical sciences.
The awesome power of modern technologies for cutting, patching, sequencing, synthesizing, making millions of copies of a specific piece of the genome and the availability of dedicated computer programmes lead to the completion of the sequencing of the human genome and that of other organisms (The Human Genome Project: International Human Genome Sequencing Consortium, 2001, 2004 and Dhand, 2006).

During the last two decades, rapidly emerging technologies used in the identification, characterization and analysis of genes have been made possible by advances in bioinformatics. Easy and immediate access to genetic data through the Internet (www; world wide web) gave a real pace to these advancements. We do not have to wait long to find out whether a gene has been mapped and cloned, which genes are present on a particular region of a chromosome, which new regulatory factors are found in gene expression and what the new prospects are in therapies for genetic disorders. Primary genetic information such as DNA sequence of various organisms, genetic maps and information about genes and their protein products are stored in dedicated and up to date databases, which are accessible to anyone. Through these, researchers can match any new sequence that they find with that already present on these databases. They can find out which genes are responsible for a certain phenotype and even where they stand in the evolutionary tree. For human geneticists working on inherited disorders, OMIM (Online Mendelian Inheritance in Man) developed by Victor McKusick is a worthwhile database where human Mendelian disorders are elaborately catalogued (www.ncbi.nlm.nih.gov/omim/). Another important website for the researchers is Medline/Pub-Med, where online
contents and abstracts of millions of research papers published in thousands of biomedical journals are easily available (www.pubmedcentral.nih.gov/).

With the publication of the sequence of the last human chromosome (Gregory et al, 2006), the primary goals of the Human Genome Project (HGP) have been achieved. Briefly, the aim was the determination of the sequence of all the 3 billion bases of the human genome. This was accomplished by the development of fast and efficient methods for high throughput DNA sequencing and compatible computer programs to collect, organize and interpret the enormous amount of data that was being generated by many dedicated laboratories world wide. More than 99% of the human genome sequence with an error rate of 1 in 100,000 bases is freely available in various public databases. The human genome contains 20,000-25,000 genes but the functional annotation of many genes is yet to be discovered. This is the main objective of molecular biologists in the post genome era.

Besides the HGP many other related projects were also initiated to aid the human genome project. These projects include the International Haplotype Map Project (Intl HapMap Consortium, 2003), Human Genome Diversity Project (HGDP, Cavalli-Sforza, 1990; Cavalli-Sforza et al, 1991), and many separate projects for sequencing the genome of model organisms as part of the comparative genomics efforts. These include some of the prokaryotes like *Escherichia coli* and *Haemophilus influenzae* as well as some eukaryotic organisms such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Mus musculus* etc. The study of the comparative genomics has provided vital information about the expression and function of
homologous genes. Mapping of the human homolog of genes from other organisms provide candidate genes for the study of their functions in human development and their dysfunction in causing inherited diseases.

As a direct consequence of the finished human genome sequence, molecular biology is in a transition phase from the study of the structure of genes and DNA sequence information to their functions. This area of study is called proteomics. This is the identification of the end product of genes, the proteins, and their involvement in different biochemical pathways and interactions in development, differentiation, growth and disease pathobiology. Proteomics and transcriptomics are now the main fields of investigation for the purpose of functional genomics.

The 'proteome' encompasses the entire complement of proteins present in a cell. Different proteins are translated in different cell types at different times as needed in the life cycle of a cell. The study of proteomics involves the characterization of proteins, their location, post-translational modifications and finally their specific function. Though the field of proteomics is in its infancy, tremendous developments have been made in the recent years in this field.

The 'transcriptome' constitutes the entire mRNA transcript that is transcribed from the genome. Although all the cells of any organism harbour the same genome, the genes that are expressed in a particular organ or tissue vary depending on its function. mRNA, rRNA and tRNA have been known to have a key role in transferring genetic information through generations. Recently a new class of small units of RNA molecules were discovered that are thought to have a
role in gene expression and gene regulation. These are micro (miRNA), small interfering (siRNA) and short hairpin (shRNA) RNA molecules.

Proteomics is much more complicated than genomics because multiple forms of proteins can be encoded by a single gene using alternative splice sites, by exploiting different reading frames and by virtue of having more than one translation initiation and termination sites. The post-translational modifications in the proteins also complicate the processing of the end product. An individual has an identical genome in almost all the cells in the body, but the total contents of the transcriptome and proteome vary significantly in different cell types and in different cellular environments according to the specific requirements, location or function of the cells. These make the field of 'functional genomics' a very dynamic and rapidly growing area in biology. Functional genomics has been benefited by the advancement of novel experimental techniques to investigate gene structure and function and the use of bioinformatics.

Modern biology and genetic medicine have grown exponentially in the last few decades and details would be beyond the scope of this presentation. Therefore, only topics relevant to the work described in this thesis will be covered briefly.

The human genome is composed of three billion bases that are packaged in 23 pairs of the human chromosomes. Twenty-two pairs of chromosomes are called 'autosomes' as they are the same in both males and females. One pair, the sex chromosome, is different. Human females have two X chromosomes while males have one X and one Y chromosome.
The genetic information carried by DNA does not get incorporated directly into proteins, which are the major structural and functional end points of this process. DNA always remains in the nucleus of the cell and is transcribed into mRNA. The mRNA migrates into the cytoplasm and is translated into polypeptide chain and proteins. A triplet of nucleotides code for an amino acid and therefore there are 64 possible combinations of the four bases that code for 20 different amino acids. It was also established that there are “start” and “stop” codons and because many different codons can code for the same amino acid, the code is redundant (Khorana, 1977).

In humans only 2-3% of the DNA codes for the proteins, while the remainder, collectively known as “junk” DNA contains non-coding regions, regulatory sequences, repetitive sequences and pseudogenes etc. A gene is a stable entity and transfers its information from generation to generation in a specified manner. Due to environmental mutagens or errors in DNA replication and repair, the base composition changes and as a result of the mutation a new allele of the gene appears. The new allele is as stable as its older version and occupies the same position on the chromosome. The frequency of mutations generally lies between one in a hundred thousand and one in a million bases. Some mutations result in structural changes in the protein product that cause an inherited disease.

In many instances, clinicians observed a disease phenotype, but the discovery of its genetic defect took half a century or more. An example is sickle cell anaemia. In 1904, James Herrick came across a case of severe anaemia and observed that the patient’s blood- smear contained red cells that were “thin,
elongated, sickle-shaped and crescent shaped”. Several years later he reported that some unrecognized change in the composition of the corpuscle itself might be the determining factor (Herrick, 1910). After years of efforts by researchers, Linus Pauling concluded that the “determining factor” was haemoglobin. Using electrophoresis to examine the physical-chemical properties of haemoglobin from normal individuals (HbA) and sickle-cell patients (HbS), Pauling et al (1949) revealed “a clear case of a change produced in a protein molecule by an allelic change in a single gene”. Ingram (1957) finally sequenced the β-chain of haemoglobin and showed that the glutamic acid at position 6 of normal HbA was replaced by valine in the HbS molecule. This results from a change in a single base, GAG to GTG in the gene. Thus, sickle cell anaemia became the first genetically transmitted disease to be characterized at the molecular level.

Because the physiological and biochemical cause of sickle cell anaemia was known before the gene was identified, it is an example of forward genetics. However, in instances when the biological defect is not know then, as described later, procedures such as linkage analysis are used to find the position of the disease-causing gene on a particular chromosome and the function of its protein product. This procedure is called reverse genetics and was used, e.g., for mapping and identifying the genetic defect that is responsible for cystic fibrosis (Riordan et al, 1989). Using these approaches, more than 1700 human disease genes have now been identified and there has been an exponential increase in the rate at which single-gene disorders are being discovered.

One of the major concerns of modern molecular biologists is to be able to find the cause of incurable diseases and their treatment. Genetically inherited
diseases that are caused by mutations in genes require detailed information about the gene, its exact location in the genome, the nature of the mutation(s) as well as the function of the final product of the gene. Applications of some approaches like linkage mapping, positional cloning and mutation screening of candidate genes have been very successful in identifying and characterizing disease-causing genes.

Ocular disorders affect a large proportion of mankind and have been of special interest to many geneticists. A great deal of work has been carried out to find the genes responsible for these diseases as a prelude to formulating therapies for their cure. More than a hundred genes and their disease causing mutations have been found to be responsible in the aetiology of eye disorders. With special relevance to the work presented here, some of the strategies applied in this area are briefly described below. Detailed accounts can be found in the Annual Reviews of Genomics and Human Genetics and textbooks, particularly Strachan and Read (2004) that gives a comprehensive and detailed description of all aspects of cell biology and genetics that are relevant to human inherited diseases.

11.1 APPROACHES AND STRATEGIES IN THE IDENTIFICATION OF DISEASE CAUSING GENES:

The term 'disease causing gene' is not an appropriate term to define a gene or its function. It is the expression of the gene that defines a particular phenotype and its malfunction due to a mutation that is responsible for causing the disease. Many different approaches have been successfully applied in the identification of disease causing genes over the past two decades. The ultimate
goal of all these strategies is to identify an appropriate 'candidate gene' and to screen/sequence it for the disease causing mutation.

1.1.1 LINKAGE BASED STRATEGIES FOR THE IDENTIFICATION OF DISEASE CAUSING GENES:

According to the Mendel's law of independent assortment, two loci that are not on the same chromosome segregate independently. Two loci that are physically located close to each other on the same chromosome (syntenic) tend to be inherited together more often then those that are far apart. Compared to loci that are close together, there is a greater chance of the physical exchange of DNA material (crossing over) between two parental loci located distant to each other on the same chromosome. As a result of crossing over, the chromosomes recombine to give a different allelic order. This is the principle of linkage analysis. The basis of linkage analysis is to determine whether the two loci co-segregate more often then they would if they were far apart on the same chromosome. Chromosomes have certain hotspots where 95% recombinations occur. These are usually 1-2 kb long and are localized within a conserved block of 20-50 kb long (Jeffreys et al, 2001). The rate of recombination between two genetic loci is directly related to the distance between the two on the same chromosome. The recombination fraction represented by 'θ' is the probability of recombination events occurring between the two loci. The values of the recombination fraction range from θ = 0 (for loci in close proximity to each other) to θ = 0.5 (for loci that are far apart or on different chromosomes). This recombination fraction is a measure of the distance that separates the two loci. The unit of map distance is a centiMorgan (cM) which is equal to 0.88 Mb. One cM represents a 1% probability
of recombination during meiosis or gamete formation which would mean that \( \theta = 0.01 \). Loci having a recombination fraction \( \theta < 0.5 \) are considered to be genetically linked, and the phenomenon is known as genetic linkage. To analyse the genetic linkage between the two loci, a test is formulated in terms of Log of the ratio of likelihood whether the two loci are linked or not. This is called a LOD score. A positive value of \( Z (\theta) \) is indicative of linkage, while negative value indicates no linkage. A value of +3 is accepted as significant evidence of linkage (Ott, 1991; Terwilliger and Ott, 1994).

Linkage analysis has been extensively used over the last two decades in localizing the genes responsible for many Mendelian diseases. This was achieved by analysing the linkage between the locus harbouring the disease causing gene and a marker, which has a precisely known genetic location. Accessibility to highly accurate human genome sequence data has identified thousands of such informative markers that include microsatellites. For the purpose of linkage analysis, an informative marker must have high polymorphic information content (PIC) and a high heterozygosity value. After establishing significant linkage of the disease locus to a known polymorphic marker, many other tightly linked markers with high heterozygosity are analysed to narrow or define the critical disease region. The next step is to sequence the candidate genes in the narrowed region of the chromosome and to pinpoint the exact disease-causing mutation.

Gene mapping experiments through linkage analyses were first successfully carried out in the fruit fly by Morgan et al (1925) and as mentioned earlier long before that, Garrod (1902) reported the Mendelian pattern of
inheritance for alcaptonuria. After the successful application of linkage analysis in fruit flies, Morton (1955) proposed the application of LOD score analysis for human linkage studies, while Elston and Stewart (1971) described the first algorithmic formulation for the analysis of large pedigrees. Automated calculation of likelihood and LOD score was presented by Ott (Ott, 1974). These early landmarks set the field for the gene identification using linkage analysis. Mapping of the hypercholesterolemia gene (Berg and Heiberg, 1978), the gene for Huntington's disease (Gusella, 1983) and the gene for cystic fibrosis (Tsui et al, 1985) were among the first genes that were mapped using this approach. The first attempt in the identification of retinal dystrophy causing genes was the localization of a gene for X-linked retinitis pigmentosa (RP) to Xp (Bhattacharya et al, 1984) and the gene for rhodopsin at 3q causing adRP (McWilliam et al, 1989; Farrar et al, 1990). These findings were the beginning of the molecular genetics of retinal degenerations.

1.1.2 GENETIC MARKERS ARE ESSENTIAL TOOLS IN DISEASE GENE MAPPING:

An important aspect in characterizing a disease-causing gene is to find exactly where it resides on a chromosome. A comprehensive map is required showing the order and relative positions of all the genes or markers. The generation of polymorphic genetic markers such as Restriction Fragment Length Polymorphism (RFLP's), microsatellites and very recently SNP's have revolutionized the identification and localization of human disease gene mapping as an integral part of the human genome project. Easy typing on a large scale by polymerase chain reaction (PCR) and sufficient distribution of these genetic
markers throughout the genome has made linkage based genetic mapping of the disease genes a very popular method among the researchers.

RFLP's were initially used for mapping disease-causing genes. Typing RFLP's was a tedious process but became simplified by the advent of PCR procedures. Apart from the time and hard work required to do the typing through RFLP, their less informativeness was a great disadvantage.

DNA minisatellite markers (variable number of tandem repeats; VNTR) were developed in mid 1980's. They are moderately long arrays (about 11-65 bp unit length) of repeated DNA sequences that tend to cluster near the telomeres. They were usually typed by Southern hybridization and were popular due to their informativeness and high heterozygosity. However, these sequences are too long to amplify by PCR and their preferential subtelomeric localization limit their application in genome wide linkage studies.

Microsatellite markers have been the most successful tools for the linkage based gene mapping. Like minisatellites, these are arrays of tandem repeats that are highly variable but smaller in size, usually less then 10 bp (Weber, 1990). Multiple microsatellite alleles are generally thought to arise from replication slippage. The new alleles are different from their progenitor alleles by the number of repeat units (Hile and Eckert, 2004). They account for about 3% of the genome. There are more than one million microsatellites in the human genome and their exact position on each chromosome is known by virtue of unique sequences of their flanking nucleotides. Di-nucleotide repeat arrays are the most common type and constitute about 0.5% of the genome. Tri and tetra repeats are less common, while penta and hexa repeats are rare but often are highly
polymorphic (International Human Genome Sequencing Consortium, 2001). The
majority of simple repeats are found in the non-coding DNA, either in the
intergenic sequence or in the introns (Ellegren, 2004) and because they are
shorter in length, they are very conveniently typed by PCR. Primers are designed
for the specific flanking sequences of the repeats and the size of the PCR
product (alleles differed by the number of repeat units) is checked by size
fractionation by polyacrylamide gel electrophoresis.

The advent of multiplex PCR has made mapping procedures very quick
and easy. In multiplex PCR, sets of compatible microsatellite markers with non-
overlapping allele size are amplified in a single tube and labelled with fluorescent
dyes for automated genotyping. With the availability of highly dense microsatellite
maps of the human genome, there has been a widespread application to linkage
mapping, paternity testing, and forensics.

Single nucleotide polymorphisms (SNP's) are the modern generation of
genetic markers that are now extensively used in mapping studies. The
advantage of SNP's is that they allow ultra high throughput genotyping and very
high marker densities (Wang et al, 1998). Higher marker densities are needed in
fine mapping in genetic linkage studies of disease genes as well as mapping of
susceptibility loci in complex diseases. SNP's are found very uniformly and
frequently in the genome. According to a recent report, human genome contains
ore than 27 million SNP's, of which, more than 4 million lie within genes (Serre
and Hudson, 2006).

1.1.3 POSITIONAL CANDIDATE GENE APPROACH FOR THE
IDENTIFICATION OF DISEASE CAUSING GENES:
After the establishment of linkage and defining the disease interval, the next step is to find appropriate candidates in the specified region. As our knowledge of the human genome advances, it becomes easy to construct a detailed map of all the definite and possible genes in the region. All those genes located or mapped within the disease interval become possible candidates. This can be termed as 'positional candidate gene approach'. The screening of all the genes reside in the region is usually not possible in all the cases. For mutation detection, there are certain criteria for a gene to be selected as candidate. The genes that have specific expression in the cell or tissue involved or a function relevant to the biochemical pathway that leads to the disease phenotype are considered first. One good example of this approach is the gene for photopigment rhodopsin. Rhodopsin was cloned and mapped on chromosome 3q21-qter (Nathans and Hogness, 1984). Linkage analysis of RP phenotype in an Irish family mapped the disease locus at the same 3q region harbouring rhodopsin gene (McWilliams et al 1989). Subsequently mutation screening of the rhodopsin gene revealed mutation associated with retinitis pigmentosa (Dryja et al, 1990). There are some cases where disease genes have been identified with the help of model organism(s). After the identification of a disease causing gene in an animal model, human homolog becomes a natural candidate. Examples of this approach are CRB1 and SEMA4A genes. Mutations in the Drosophila crumbs gene have been reported to cause photoreceptor degeneration (Tepass et al, 1990). A locus for human RP12 was mapped to chromosome 1q31 (van Soest et al, 1994). CRB1 is the human homolog of Drosophila crumbs gene and mutations in this gene subsequently reported to cause RP and LCA (den
Hollander et al, 1999, 2001). Sema4A gene reported to cause severe retinal degeneration in a mouse model (Rice et al, 2004). Without any prior linkage or positional confirmation, human SEMA4A gene was considered as candidate for human retinal degeneration. Mutation screening of SEMA4A gene in a panel of patients revealed several mutation causing CRD and RP (Abid et al, 2006). Additionally, if a gene in the disease causing region has a close functional relationship to a gene already known to be involved in a similar disease phenotype. RPGR gene has been known to cause X-linked retinitis pigmentosa (Buraczynska et al, 1997). RPRIP1 gene was found to interact with RPGR protein in the photoreceptor connecting cilia (Boylan and Wright, 2000; Hong et al, 2001). Subsequently mutations in the RPGRIP1 gene were found to cause Leber congenital amaurosis (Dryja et al, 2001). Exceptions to these rules include the splicing factor genes that are ubiquitously expressed in the body but have been found to be responsible for the disease phenotype only in a specific tissue that is diseased, such as the retina (McKie et al, 2001; Vithana et al, 2001; Chakarova et al, 2002).
1.2 THE EYE:

1.2.1 ANATOMY OF THE EYE:

The human eye is a complex sense organ that develops directly from the central nervous system. It is protected by the bony eye socket posteriorly and the eyelids and eyelashes anteriorly (Figure 1.1). The diameter of a normal adult human eye is 22-23mm. Because the two eyes are physically separated, they transmit two slightly different images to the brain. In the brain the two images are fused together to give a three dimensional or stereoscopic image. The focusing system of the eye includes the cornea and the crystalline lens.

The cornea is a flattened dome shaped front covering of the eye. It is about 1.2mm thick at the periphery and 0.5 – 0.6mm at its centre. It also serves as a filter for the infrared and ultraviolet lights that are harmful to the lens and the retina. It is richly supplied with nerve fibres but is devoid of blood supply. Thus it receives its nourishment from the aqueous humour and the limbal blood supply at the junction of cornea and sclera.

The sclera is located behind the cornea. It is a white, collageneous wall that overlaps the cornea all round the periphery and is surrounded by the uveal tract on its inner side. The uveal tract consists of three parts that are choroid, ciliary body and iris. Of these, the choroid and ciliary body line the sclera and a free circular diaphragm, the iris, is situated anteriorly. Pigmentation in the iris reflects the colour of the eye. Dense pigmentation colour the eye brown while light pigmentation gives the irides a blue colour. The iris has a central flexible aperture known as the pupil that regulates the amount of light entering the eye by the constriction and expansion of muscles. The ciliary body is the continuation of
the choroids and acts as the suspension of the lens. Ciliary glands found in the
ciliary body are involved in the secretion of the aqueous humour that fills the
cavity between the cornea and the lens. The aqueous humour is mainly
responsible for the constancy of the optical dimensions of the eyeball by
maintaining the intra-ocular pressure. It is an isotonic solution that fulfils the
nutritional requirements of the corneal endothelium. It absorbs the waste
products and the remaining portion of the light of short wavelength that has
passed through the cornea. The lens is an asymmetric convex body, about
10mm in diameter enclosed in a capsule. It is completely devoid of nerve fibre or
blood supply and held in place by suspensory ligaments or zonular nerve fibre.
Behind the lens, the ciliary body is continuous with the vitreous humour and
secretes some of its components.

The choroid is a vascular layer in loose contact with the sclera, leaving the
epichoroidal space. The choroid is richly supplied with arterioles, a spongy
capillary layer, large draining veins and sensory nerve fibres. Cells of the choroid
contain melanin to give the inner part of the eye a dark appearance. It serves to
improve the visual process by absorbing the light to avoid internal reflection. The
vitreous humour fills the large cavity between the lens and the sensory layer of
the retina. Vitreous humour is a transparent gel composed of water and
transparent remnants of embryological vascular system that has provided the
nutrients during the development of the eye.
Anatomy of the Human Eye

Adapted from http://en.wikipedia.org/wiki/eye
1.2.2 THE RETINA:

The retina is the inner most neuro-sensory layer of the eye. It is part of the brain through which the reception and transmission of the light stimuli occurs. On its internal side it is in contact with the vitreous body while externally it is attached firmly with the Bruch’s membrane, which separates the retina from the choroid. Retina is continuous with the optic nerve, which enters into the retina from the nasal side known as the optic disc. The optic nerve is about 1.5 mm in diameter and insensitive to light thus referred as blind spot. From the centre of the optic disc central retinal artery and vein enter the retina, which are the main source of nourishment to the inner retinal surface. However the outer retinal lining and the photosensitive elements mainly depend on the choroidal blood supply for their nutritional requirements. Structurally the retina is divided into two layers. A sensory layer (neuronal retina) and a supportive layer (retinal pigment epithelium) both are derived from the inner and outer layers of the optic vesicle respectively. The consistency of the neuronal retina is not the same throughout the eye globe. The central part of the retina is known as the macula that is responsible for sharp vision. It has a central depression known as the fovea where the retina becomes exceedingly thin. Some of the layers of the retina pull aside from the fovea to provide maximum resolution of the image. Blood vessels are also absent from the entire macula to decrease the interference with the incoming light transmission.

1.2.3 THE NEURONAL RETINA:

The neuronal retina consists of photoreceptor cells (rods and cones), outer limiting membrane, outer nuclear layer, outer plexiform layer, inner nuclear
layer, inner plexiform layer, ganglion cell layer, layer of optic nerve fibre and the inner limiting membrane. The outer layer of the neural retina contains photoreceptor cells. The inner layer contains bipolar cells, horizontal and amacrine cells (Figure 1.2). These cells synapse with the photoreceptors and process the visual information in the outer and inner retina. Ganglion cells are the output units that form the innermost layer of the retina. Their long axons track along the inner surface of the retina and collectively form the optic nerve. Among these neuronal cells, photoreceptors are of great importance because they provide the mechanism through which the light energy is converted into neural impulses. All the other neurons (bipolar cells, ganglion cells) together form a pathway for the conduction of these neural impulses all the way to the visual cortex of the brain. In addition the retina contains neuroglial elements (Muller cells). They provide nutrition and mechanical support to the sensory retina (Ripps and Wilkovsky 1985).
Figure 1.2

Schematic representation of the layers of the retina

Adapted from lectures of Dr. Paul Patton: Visual System I.
http://soma.npa.uiuc.edu/courses/bio303
Photoreceptor cells are of two types: the rods and the cones (Figure 1.2). They have four functional segments: the outer segment (OS), the inner segment (IS), the nucleus and the synaptic body. The OS has a microtubule-based cytoskeleton known as axoneme, which starts from the IS, runs through the interconnecting cilium and extends into the OS up to half of its length (Kaplan et al, 1987). The connecting cilium bridges the outer segment with the rest of the cell body of the photoreceptor.

The OS of both rods and cones have specialized flattened invaginations of plasma membrane stacked to make discs like structure along the axis of the axoneme. In the outer segments there are more than 1,000 discs that harbour the photochemical pigment. New discs continually replace old discs by a process of plasma membrane invagination at the base, adjacent to the axoneme. Older discs are shed at the top of the OS and phagocytosed by the cells of the retinal pigment epithelium (Anderson et al, 1978; Boesze-Battaglia and Goldberg, 2002). The photochemical or visual pigment in the photoreceptor cells consists of a membrane protein (opsin) and a chromophore (a vitamin ‘A’ derivative). All the other organelles that are required in the normal functioning of the cell are housed in the inner segment of photoreceptor cells.

There are slight differences in the morphology and functions of the two photoreceptors. Rods are thin cylindrical structures that provide monochromatic vision and operate under dim light (mesopic) and in the darkness (scotopic). However the cone photoreceptors as the name describe are conical in shape and activate under the conditions of bright light (photopic) and provide trichromatic (colour) vision. There are three types of cones depending upon the nature of the
light sensitive pigment (blue, green and red) they contain. The rod cells are 300 times more sensitive and are also more abundant in number than the cones. An estimated 100,000,000 rods and 3,000,000 cones occupy the entire retina. The collective signals of approximately 200 rods converge on the same nerve fibre that transmits more intense stimuli through the peripheral ganglion cells. Cones are densely populated in the region of fovea. The high intensity of cones in the foveal region corresponds to an equal number of nerve cells. This explains the high degree of visual acuity in the central retina in comparison with poor visual acuity at the periphery. However the peripheral retina has a greater sensitivity to weak light because of the abundance of rods that are more sensitive to dim light.

1.2.4 RETINAL PIGMENT EPITHELIUM:

The retinal pigment epithelium (RPE) is the non-neural lining of retina and consists of a monolayer of 4-6 million epithelial cells. Each RPE cell has microvilli on its surface. On the basai side, these villi are attached to the Bruch’s membrane of the choroid and allow the transport of oxygen, glucose and other metabolites from the chorio-capillaries to the neural retina, in particular the photoreceptors. The RPE provides a physical barrier for the absorption of molecules from the highly vascular choroid to the sensory retina. Apical microvilli are in loose contact with the outer segments of the photoreceptors where they are actively involved in the renewal and replacement of the outer segment discs through phagocytosis. RPE cells are said to be the most phagocytic cells in the human body as they daily engulf 10% of total mass of each outer segment of photoreceptors. Each of the RPE cells is in contact with approximately 45 photoreceptor cells. The space between the photoreceptors and the villi of the
epithelial cells are filled with the interphotoreceptor matrix. The cytoplasm of the RPE cells contain a large number of melanin pigment granules that control the reflection of light by absorbing the extra amount of light and thus provide an anti reflective coating for the retina. Another important function of the RPE is that it is responsible for the regular processing and transport of vitamin 'A' derivatives (retinoids), which are an intermediate in the visual cascade (La Cour, 2003). It is also involved in the secretion of the interphotoreceptor matrix and the maintenance of the photoreceptor microenvironment.

1.3 PHOTOTRANSDUCTION:

As described earlier, the human retina has two types of photoreceptor cells, the rods and the cones. The rods function in dim light but have no colour perception. The cones, on the other hand, function in bright light and are capable of colour perception. Vision is initiated in the outer segments of the rod and cone photoreceptor cells. The photosensitive molecule in the discs of the photoreceptors is rhodopsin. It is a member of G-protein coupled receptor family (GPCR) and comprises of 7-transmembrane helices. It is composed of an apoprotein called opsin, with 11-cis retinal, an efficient chromophore, as a prosthetic group that gives rhodopsin its rose colour and photon absorbing ability. It is attached to opsin by a Schiff’s base bond- the aldehyde group of this polyene chromophore is linked to the ε-amino group of a specific lysine molecule in the opsin (Wald, 1968).
The process of vision starts with the absorption of a photon of light by the rhodopsin. This initiates a cascade of enzymatic cascade that converts the electromagnetic stimulus into a biochemical messenger and ultimately into a neurological excitation and visual cortex stimulation (Figure 1.3; Dowling, 1987; Wu and Maple, 1998; Baehr and Liebman, 2001).

The cascade can be summarized as follows: Photoexcitation in the rod cells starts with the photo-isomerization of rhodopsin that in turn cause activation of rod transducin. This activated transducin subsequently stimulates the phosphodiesterase (PDE) mediated hydrolysis of cyclic 3',5' GMP (cGMP). Due to this rapid decrease in the cGMP level, the cGMP-gated channel closes thus blocking the influx of Na\(^+\) and Ca\(^{++}\) ions. This leads to the hyperpolarization of the plasma membrane and a reduced release of glutamate neurotransmitter at the synaptic end of the photoreceptor. This drop in the Ca\(^{++}\) influx into the photoreceptor cells stimulates the guanylyl cyclase activating protein (GCAP). This in turn activates retinal guanylyl cyclase (RetGC) that restores cGMP levels by converting GTP to cyclic 3',5' cGMP. This step initiates the opening of cGMP-gated channels and the recovery of the dark adapted state. In short, the light-adapted state represents closed cGMP gated cation channels, whereas the dark-

Light consists of a stream of electromagnetic particles called photons. Although both rods and cones respond to a photon, the rods have a 100-fold higher cellular amplification and duration than the cones. Once a photon is absorbed, its energy is converted to a chemical change in rhodopsin, i.e., sterioisomerization of 11-cis retinal to all-trans retinal. Thus the primary reaction in vision is a cis-trans isomerization of the retinal chromophore, which is highly efficient in rhodopsin (Dartnall, 1967). This is essential to make twilight vision highly sensitive. In fact the human rod cells can respond to single photon absorption. The light activated rhodopsin becomes colourless. This is called the bleaching process because rhodopsin looses its colour during activation (Kandori et al, 2001).

The photoactivated rhodopsin (R*) then binds to a G-protein, transducin. Each rhodopsin can activate more than five hundred transducin molecules. In this way a signal-amplifying process starts that continues throughout the cascade. Transducin, a member of a G-protein family consists of three subunits α, β and γ (Noel et al, 1993). R* interacts with the transducin molecule by catalyzing Tα bound guanosine diphosphate (GDP) to guanosine triphosphate (GTP). This exchange of GDP to GTP causes separation of R* from the activated transducin complex which in turn dissociates from the Tβγ subunit complex. The released R* then continuously activates other transducin in the same way. Tα-GTP complex activates the enzyme cGMP phosphodiesterase (PDE) in the
cytoplasm. PDE comprises of two catalytic $\alpha$ and $\beta$ subunit and two inhibitory $\gamma$ and $\gamma'$ subunits. Activated $T_\alpha$-GTP interacts with $\gamma$ subunits of PDE and removes them from the catalytic $\alpha$ and $\beta$ subunits. This interaction results in the hydrolysis of 3', 5' cGMP to 5'-GMP and $T_\alpha$ dissociates from the $T_\alpha$GTP complex. Due to this rapid hydrolysis, the level of cGMP decreases very rapidly resulting in the closure of the cGMP-gated Na$^+$/Ca$^{++}$ channel, which controls the transport of ions across the photoreceptor plasma membrane. The hyperpolarisation of the plasma membrane due to the decrease in the conductance of Na$^+$, Ca$^{++}$ cations result in the reduced release of the rod's neurotransmitter, glutamate, to the adjacent neurons, signalled by the synaptic termini. The synaptic ends of the photoreceptors synapse with the horizontal and bipolar cells in the outer plexiform layer. Horizontal and bipolar cells synapse with the ganglion cells in the inner plexiform layer.

In order to restore the dark-adapted state, the photoactivation response has to be terminated. This requires the inactivation of $R^*$ and $T_\alpha$, reassociation of the inhibitory PDE$\gamma$ subunit to the catalytic PDE$\alpha\beta$ subunit and the stimulation of retinal guanylate cyclase (RetGC) to increase the cGMP levels in the photoreceptors.

To inactivate $R^*$, the phosphorylation of serine and threonine residues in the carboxy-terminus of $R^*$ is catalysed by rhodopsin kinase (RK). This phosphorylated rhodopsin subsequently binds to arrestin (Arr) that reduces its ability to further activate the transducin molecule. Inactivation of the transducin molecule takes place by the activity of GTPase activating protein (GAP). This
protein hydrolyses the GTP that leads to the dissociation of the $\alpha$ subunit from the $\gamma$ subunit. The $\gamma$ subunit subsequently reassociates with the $\alpha\beta$ subunit, thus blocking its activity. The regeneration of the rhodopsin molecule takes place by recombining rhodopsin with 11-cis-retinal.

To depolarise the cell, high levels of cGMP are required to keep the GMP gated channels open and the normal release of glutamate neurotransmitter. Ca$^{++}$ ions play an important role in the recovery of the dark-adapted state. The extracellular Ca$^{++}$ enters through the open cGMP-gated channels and is balanced by its outward flow through the Na$^{+}$/ Ca$^{++}$ exchangers in the outer segment plasma membrane. Upon the closure of the cation channels by photoactivation, their efflux through the exchangers continues resulting in the decrease in intracellular Ca$^{++}$ levels. The guanylate cyclase activating protein (GCAP) binds three Ca$^{++}$ molecules in the dark state that has an inhibitory effect on the activity of GCAP protein. After the fall in the intracellular Ca$^{++}$ levels, Ca$^{++}$ dissociates from the GCAP protein returning it to an active state. These activated GCAP proteins subsequently activates retinal guanylate cyclase (RetGC) which catalyses the synthesis of cGMP from GTP and thus the gated channels are opened.

Therefore, the photoactivation of rhodopsin leads to changes in the net production of cGMP by perturbing two opposing catalytic activities: degradation by phosphodiesterase (PDE) and synthesis by guanylate cyclase (RetGC). Because cGMP levels govern how many cation channels are open, the light signal is transformed from a physical stimulus to a biochemical chain of reactions
that culminates in the hyperpolarization and the depolarization of the plasma membrane (Ridge et al, 2003).

So far, the understanding of the phototransduction cascade represents the best understood G-protein coupled receptor (GPCR)-mediated signal transduction. Most of the major components and pathways have been identified through biochemical and molecular biological approaches. Still much work remain to be elucidated to explain that how the absorption of a single photon leads to changes in the neuronal transmission. This understanding will also further elaborate the mechanisms leading to retinal degeneration.
1.4 HEREDITARY RETINAL DYSTROPHIES:

Hereditary retinal degenerations are classically defined by the age of onset, severity and patterns of visual loss (peripheral or central i.e. involvement of rods and cones) ophthalmoscopic and electrophysiological examinations and family history. They are generally classified according to the mode of inheritance that are autosomal dominant (adRP), autosomal recessive (arRP), X-linked (XLRP), mitochondrial and digenic.

With an estimated worldwide prevalence of 1 in 5,000 (Weleber and Gregory-Evans, 2001), retinitis pigmentosa (RP) is the most prevalent type of progressive retinal degenerations that bilaterally affect rods and cones simultaneously.

A person affected by retinitis pigmentosa primarily suffers from night blindness and loss of peripheral vision mainly because of the degeneration of the rod cells, which are responsible for the light perception in the dark and are abundant in the peripheral retina. With the gradual loss of rods, cone cells start degenerating due to some unknown apoptotic mechanism and eventually the central vision is also lost in advanced stages of the disease (Berson, 1993; 1996).

In some cases of retinal degenerations, the cone cells die first followed by the degeneration of rods. This type of retinal degeneration is referred to as cone-rod dystrophy (CRD). The difference between the two types is the rapid loss of either rods and/or cones respectively. Especially at the later stages of disease, when the patient is completely blind, it is hard to distinguish between the two phenotypes (Rivolta et al, 2002). The most severe type of retinal degeneration is
Leber's congenital amaurosis (LCA). In this case both rods and cones are dead at birth or early in infancy (Francois, 1968).

The heterogeneity in retinal degenerations (including LCA, RP and CRD) is well documented both clinically and genetically. Various clinical phenotypes can be caused by different mutations in a single gene and many different genes are reported to cause the same phenotype. This suggests that there are a number of possible origins of disease pathology, all of which result in the same degenerative process. They occur as a result of a plethora of different mutations in a large number of genes (Table 1.1). Approximately 30% of the patients with adRP have mutations in RHO or RP1 gene (van Soest et al, 1999). RDS and CRX gene mutations represented only 4.5% and 0.4% of RP respectively in a large survey (Sohocki et al, 2001). A detailed mutation screening of the genes involved in LCA was also carried out by Hanein et al (2004) that showed mutations in GUCY2D to be the most prevalent (21.2%) cause, while CRB1 found to be responsible for 10%, RPE65 for 6.1%, RPGRIP1 for 4.5%, AIPL1 for 3.4%, TULP1 for 1.7% and CRX for 0.6% of all LCA cases.

1.4.1 CLINICAL DESCRIPTION OF RETINITIS PIGMENTOSA:

In classical RP night blindness starts in early childhood and remains the only symptom for many years before other symptoms, if any appear. Due to the early loss of photoreceptor cells in the peripheral retina, the field of vision becomes constricted to the tunnel vision. At advanced stages of disease the central vision is also lost and patients become completely blind (van Soest et al, 1999; Heckenlively and Daiger, 2002). Electroretinographic (ERG) changes can be observed in very early stages of the disease, as early as a decade before
other diagnostic symptoms appear on ocular examination (Berson, 1993, 1996). In the early stages, patient's ERGs have reduced amplitude. As disease progresses the ERG amplitude becomes even smaller.

The earliest ophthalmoscopic signs are 'bony spicule' type pigmentary deposits that migrate from the RPE and spread initially along the equatorial region of the retina (Heckenlively, 1988). These pigmentary deposits tend to spread towards the periphery, sparing the macula until late in the disease. Another important ophthalmoscopic finding is the attenuation of blood vessels especially the arterioles, while the pigmentation along these arterioles hides their course giving a spidery outline. Due to the discoloration of the RPE, the choroidal vesculature becomes visible through the fundus. The optic disc becomes atrophic, giving yellow or pale waxy appearance.

1.4.1A THE GENETICS OF RETINITIS PIGMENTOSA:

The genetics of RP is complex, approximately 50% of the RP cases are sporadic or simplex and most of them are recessive in nature (Jay, 1982). Autosomal dominant and autosomal recessive each account for about 20% of all reported RP cases, while 10% cases are of X-linked RP (Wang et al, 2001). Only two genes (RDS and ROM1) causing digenic RP have been reported. This is the first example that demonstrates the 'two locus mechanism' where the mutations in two different genes are responsible to cause a disease in human (Kajiwara et al, 1994). RP also appear as an associated anomaly in more than 30 syndromes, the common examples are Usher syndrome, Bardet-Beidl syndrome and Refsum disease (De Jong et al, 1991; Pagon, 1993). Most of the syndromes show recessive mode of inheritance.
More than 100 different genes have been reported to cause a variety of retinal degenerations using approaches such as physical mapping and positional cloning to identify the disease causing genes. A comprehensive collection of references for retinal degenerations can be obtained from RetNet (http://www.sph.uth.tmc.edu/RetNet). The genetic causes in 60% of RP cases are still unknown (Wang et al, 2005). Due to the disease heterogeneity, the identified genes are of very diverse in nature and many of these are responsible for more than one phenotype. A large number of mutations in these genes are reported to cause retinal degeneration. The function of most of these genes and the mechanism by which the identified mutations lead to the death of photoreceptors cells are still poorly understood.

RHO, RPGR, RP1 and RPE65 are among those genes, which have been studied thoroughly. They also contribute the largest number of known mutations causing RP that describe the importance of these genes in the mechanism of vision. It is also a fact that no single mutation in all the known genes alone accounts for 10% of the unrelated RP cases (Wang et al, 2005). Recently the involvement of pre-mRNA splicing factor genes, PRPC8, PRP31 and HPRP3 in the pathogenesis of RP has been reported (McKie et al, 2001; Vithana et al, 2001; Chakarova et al, 2002). It is interesting to know that though these are widely expressed genes, they only cause pathological changes in photoreceptor neurons. These findings depict the importance and sensitivity of photoreceptors and functional diversity of the genes causing RP.
1.4.2 CLINICAL DESCRIPTION OF LEBER CONGENITAL AMAUROSIS:

LCA accounts for about 5% of all inherited retinopathies and responsible for 10-18% of congenital blindness (Kaplan et al, 1990; Graw, 2003). Electroretinography is an important criterion in the diagnosis of LCA, there are no detectable ERG responses from either rod or cone photoreceptors suggesting early or severe impairment of both photoreceptor systems. Fundoscopy shows variation of retinal morphology from relatively normal to signs of pigmentary retinopathy (Harris, 2001). Other ophthalmologic signs presented in LCA are sluggish papillary response, infantile nystagmus, roving eye movement, squint, occasional photophobia, cataract, keratoconus and keratoglobus. LCA phenotype is also reported to be associated with many syndromic anomalies of the renal, cardiac, skeletal and central nervous system. Among the associated symptoms, mental retardation seems to be an important feature. It is found in around 20% of the LCA population (Fazzi et al, 2003).

1.4.2A GENETICS OF LEBER CONGENITAL AMAUROSIS:

LCA generally inherited in an autosomal recessive manner, but have also been reported in some dominant families (Leber, 1869; Heckenlively, 1988). To date eight genes have been identified causing LCA. These genes are of diverse nature and are involved in a variety of biochemical functions; RPE65 and GUCY2D (RetGC) are required for phototransduction; CRX a transcription factor gene and AIPL1 are involved in photoreceptor development and differentiation. CRB1 is responsible for cell polarity while SEMA4A and RPGRIP1 are needed for photoreceptor subcellular protein translocation (Cremers et al, 2002; Abid et al, 2006). TULP1 and LRAT are also known to cause LCA but their roles in the
pathology of the disease are poorly understood. Besides these disease causing genes, two other disease loci have been identified on chromosome 14q24 and 6q11-q16, for which the genes are still unknown (Stockton et al, 1998; Dharmaraj et al, 2000).

1.4.3 CLINICAL DESCRIPTION OF CONE-ROD DYSTROPHIES:

Cone-rod dystrophy (CRD) is a progressive retinal degeneration characterized by an early loss of visual acuity and colour vision mainly due to the early loss of cone photoreceptors. As the disease progress peripheral visual field also deteriorates (Moore, 1992). CRD patients suffer from severe photophobia and epiphora usually from the first and second decade of life. Most of the patients retained some visual acuity throughout their life. ERG's show a definite cone-rod pattern in the early stages of the disease where cone ERG's are much more reduced than the rod ERG's. With the progression of the disease both the cone and rod electroretinograms become nearly equal showing the involvement of both photoreceptor system (Heckenlively, 1987). With the overlapping signs and symptoms of disease progression with other retinal degenerative diseases like RP, the absence of cone ERG response in the presence of rod ERG response generally an important criterion for the diagnosis of CRD. Fundoscopic examination reveal marked macular degeneration, significant attenuation of retinal arterioles and bony-corpuscle type pigmentary changes. Later in life the midperipheral and peripheral retina also show degenerative changes (Ismail et al, 2006).
1.4.3A GENETICS OF CONE-ROD DYSTROPHIES:

Cone rod dystrophies can be inherited in an autosomal dominant, autosomal recessive and X-linked pattern. Molecular studies have identified about 10 different loci, each of which contains a CRD-causing gene. As described earlier, enormous heterogeneity in the genetics of retinal degeneration have been reported. Mutations in ABCA4, RPRGIP1 and SEMA4A have been reported to cause autosomal recessive CRD (Cremers et al, 1998; Hameed et al, 2003; Abid et al, 2006). Mutations in CRX, GUCY2D, AIPL1, HGR4, GUCA1A and RIM1, are reported to be involved in autosomal dominant CRD (Freund et al, 1997; Kelsell et al, 1998; Sohocki et al, 1999; Kobayashi et al, 2000; Downes et al, 2001, Johnson et al, 2003,). Besides these disease causing genes and their reported mutations, certain loci have been identified for which genes remain unknown. These loci are CORD8 (1q12-q24; Ismail et al, 2006), CORD9 (8p11; Danciger et al, 2001), CORD4 (17q11.2; Klystra and Aylsworth, 1993), CORD1 (18q21.1-21.2; Warburg et al, 1991) and CORD4 (Xp11.4-q13.1; Jaikanen et al, 2003).
1.5 SOME IMPORTANT GENES CAUSING RETINAL DEGENERATIONS:

As described earlier more than hundred genes are known to cause various forms of retinal degenerations. Their protein product functions and their locations are summarised in table 1.1 and Figure 1.3. The following section describes some of the important genes that are implicated in retinal degeneration.
<table>
<thead>
<tr>
<th>Genes Involved in Phototransduction Cascade</th>
<th>References</th>
<th>Function</th>
<th>Location</th>
<th>Disease Protein</th>
<th>Disease</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>RHO</td>
<td>Dryja et al., 1990; Dryja et al., 1990a; Farar et al., 1990; Rosenfeld et al., 1992</td>
<td>Rhodopsin is a member of G-protein coupled receptor family, isomerization of rhodopsin initiates phototransduction cascade</td>
<td>3q22-q24</td>
<td>Dominant RP, recessive RP, dominant CSNB</td>
<td>180380</td>
<td></td>
</tr>
<tr>
<td>SAG</td>
<td>Nakazawa et al., 1995</td>
<td>Specifically binds to rhodopsin molecule to inactivate rhodopsin</td>
<td>2q37.1</td>
<td>Recessive RP</td>
<td>181031</td>
<td></td>
</tr>
<tr>
<td>ARRESTIN (S-antigen)</td>
<td>Retinal-specific guanylate cyclase is responsible for the generation of cGMP</td>
<td>17p13.1</td>
<td>Recessive LCA, dominant cone dystrophy</td>
<td>204000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GUCY2D</td>
<td>Guanylate cyclase enzyme is responsible for the generation of cGMP</td>
<td>17p13.1</td>
<td>Recessive RP</td>
<td>204000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDE6B</td>
<td>Phosphodiesterase hydrolyses cGMP into GMP</td>
<td>4p16.3</td>
<td>Recessive RP</td>
<td>163500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNGA1</td>
<td>Phosphodiesterase hydrolyses cGMP into GMP, channel protein that controls the transport of ions across the plasma membrane</td>
<td>4p12</td>
<td>Recessive RP</td>
<td>123825</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNGB1</td>
<td>Phosphodiesterase hydrolyses cGMP into GMP</td>
<td>5q33.1</td>
<td>Recessive RP</td>
<td>180071</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDE6A</td>
<td>Phosphodiesterase hydrolyses cGMP into GMP</td>
<td>1q13</td>
<td>Recessive RP</td>
<td>180071</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 1.1: List of Genes and Their Functions Causing Retinal Degenerations:**
<table>
<thead>
<tr>
<th>Symbol; OMIM #</th>
<th>Location</th>
<th>Disease Phenotype</th>
<th>Disease Protein</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPE65 180069</td>
<td>1p31</td>
<td>Recessive LCA, recessive RP</td>
<td>Retinal pigment epithelium-specific 65 KD protein</td>
<td>Protein necessary for the production of 11-cis-vitamin A</td>
<td>Redmond et al, 1998; Morimura et al, 1998; Marlhens et al, 1997</td>
</tr>
<tr>
<td>LRAT 604863</td>
<td>4q31.2</td>
<td>arRP, severe early onset RP</td>
<td>Lecithin retinol acyltransferase</td>
<td>Protein transforms vitamin A into 11-cis-retinol</td>
<td>Ruiz et al, 1999; Ruiz et al, 2001; Thompson et al, 2001</td>
</tr>
<tr>
<td>RDH5 136880</td>
<td>12q13.2</td>
<td>Recessive cone dystrophy</td>
<td>11-cis-retinol dehydrogenase</td>
<td>An RPE microsomal enzyme involved in the conversion of 11-cis-retinol to 11-cis-retinol</td>
<td>Nakamura et al, 2000; Cideciyan et al, 2000</td>
</tr>
</tbody>
</table>

**Genes Involved in Visual Cycle**
<table>
<thead>
<tr>
<th>Symbol; OMIM #</th>
<th>Location</th>
<th>Disease Phenotype</th>
<th>Disease Protein</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>RDH12</td>
<td>14q24.1</td>
<td>LCA</td>
<td>Retinol dehydrogenase 12</td>
<td>Protein has a dual specificity for all-trans-retinols and cis-retinols</td>
<td>Janecke et al, 2004; Perrault et al, 2004</td>
</tr>
<tr>
<td>RGR 600342</td>
<td>10q23.1</td>
<td>Recessive RP</td>
<td>RPE-retinal G protein coupled receptor</td>
<td>Binds to all-trans-retinal</td>
<td>Morimura et al, 1999</td>
</tr>
</tbody>
</table>

**Splicing Factors Genes**

<table>
<thead>
<tr>
<th>Symbol; OMIM #</th>
<th>Location</th>
<th>Disease Phenotype</th>
<th>Disease Protein</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRPF3 6014714</td>
<td>1q21.1</td>
<td>Dominant RP</td>
<td>Human homolog of yeast pre-mRNA splicing factor 3</td>
<td>Protein is a highly conserved, ubiquitously-expressed member of the U4/U6-U5 tri-snRNP particle complex</td>
<td>Chakarova et al, 2002</td>
</tr>
<tr>
<td>PRPF8 600059</td>
<td>17p13.3</td>
<td>Dominant RP</td>
<td>Human homolog of yeast pre-mRNA splicing factor C8</td>
<td>Protein is a highly conserved, ubiquitously-expressed member of the U4/U6-U5 tri-snRNP particle complex</td>
<td>Tarttelin et al, 1996; McKie et al, 2001</td>
</tr>
<tr>
<td>PRPF31 600138</td>
<td>19q13.42</td>
<td>Dominant RP</td>
<td>Human homolog of yeast pre-mRNA splicing factor 31</td>
<td>Protein is a highly conserved, ubiquitously-expressed member of the U4/U6-U5 tri-snRNP particle complex</td>
<td>Vithana et al, 2001; Vithana et al, 2003</td>
</tr>
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</table>

**Transcription Factors Genes**

<table>
<thead>
<tr>
<th>Symbol; OMIM #</th>
<th>Location</th>
<th>Disease Phenotype</th>
<th>Disease Protein</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRL 162080</td>
<td>14q11.2</td>
<td>Recessive RP, dominant RP</td>
<td>Neural retina lucine zipper</td>
<td>Interacts with CRX and promotes transcription of rhodopsin and other retinal</td>
<td>Rehentulla et al, 1996; Mears et al, 2001; Nishiguchi et al, 2001</td>
</tr>
<tr>
<td>Symbol; OMIN #</td>
<td>Location</td>
<td>Disease Phenotype</td>
<td>Disease Protein</td>
<td>Function</td>
<td>Reference</td>
</tr>
<tr>
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<td>-----------</td>
<td>----------------------------------------</td>
<td>------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>NR2E3 268100</td>
<td>15q23</td>
<td>Recessive RP</td>
<td>Nuclear receptor subfamily 2, group E3</td>
<td>A ligand -dependant transcription factor</td>
<td>Kobayashi et al, 1999; Haider et al, 2001</td>
</tr>
<tr>
<td>PIM1K; RP9; PAP1 607331, 180104</td>
<td>7p14.3</td>
<td>Dominant RP</td>
<td>RP9 protein or PIM 1-kinase associated protein 1</td>
<td>A ubiquitous protein has a role in pre-mRNA splicing and interacts with a U2 complex splice factor</td>
<td>Maita et al, 2004; Keen et al, 2002</td>
</tr>
<tr>
<td>CRX 120970</td>
<td>19q13.3</td>
<td>Cone-rod dystrophy, LCA, dominant RP</td>
<td>Cone-rod otx-like homeobox transcription factor</td>
<td>A transcription factor that interacts with NRL</td>
<td>Swaroop et al, 1999; Sohocki et al, 1998</td>
</tr>
</tbody>
</table>

### Photoreceptor Structural Genes

<table>
<thead>
<tr>
<th>Symbol; OMIN #</th>
<th>Location</th>
<th>Disease Phenotype</th>
<th>Disease Protein</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPGRIP1 605446</td>
<td>14q11</td>
<td>LCA, cone-rod dystrophy</td>
<td>RPGR-interacting protein 1</td>
<td>Protein interacts with RPGR and localizes to ciliary structure between outer and inner photoreceptor segment</td>
<td>Roepman et al, 2000; Dryja et al, 2001; Boylan and Wright, 2000</td>
</tr>
<tr>
<td>RP1 180100</td>
<td>8q11-q13</td>
<td>Dominant RP, recessive RP</td>
<td>Rp1 protein</td>
<td>Protein localizes to connecting cilia</td>
<td>Bowne et al, 1999; Khaliq et al, 2005; Liu et al, 2002</td>
</tr>
<tr>
<td>RP2 312600</td>
<td>XP11.23</td>
<td>X-linked RP</td>
<td>XRP2 protein similar to human cofactor C</td>
<td>Cofactor C protein is involved in beta-tubulin foldings</td>
<td>Schwahn et al, 1998; Mears et al, 1999</td>
</tr>
<tr>
<td>ROM1</td>
<td>11q13</td>
<td>Dominant RP,</td>
<td>Retinal outer</td>
<td>Dimers of ROM1 form</td>
<td>Bascom et al, 1995;</td>
</tr>
<tr>
<td>Symbol; OMIM #</td>
<td>Location</td>
<td>Disease Phenotype</td>
<td>Disease Protein</td>
<td>Function</td>
<td>References</td>
</tr>
<tr>
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<td>------------</td>
</tr>
<tr>
<td>APL1 604392</td>
<td>17p13.2</td>
<td>Recessive LCA, dominant cone rod dystrophy</td>
<td>Arylhydrocarbon-interacting receptor protein-like 1</td>
<td>Protein involved in nuclear transport and chaperone activity during development</td>
<td>Sohocki et al, 2000; van der Spuy et al, 2002</td>
</tr>
<tr>
<td>SEMA4A 607292</td>
<td>1q22</td>
<td>Dominant RP, dominant cone rod dystrophy</td>
<td>Semaphorin4A</td>
<td>A family of transmembrane protein involved in neuronal development and immune response</td>
<td>Abid et al, 2006; Rice et al, 2004; Kumanogoh et al, 2002</td>
</tr>
</tbody>
</table>

Genes involved in Photoreceptor development and Morphogenesis

- Dryja et al, 1997; Kajiwara et al, 1994
- Tubb et al, 2000; Wada et al, 2001
- Lotery et al, 2001a; Lotery et al, 2001b; den Hollander et al, 1999
<table>
<thead>
<tr>
<th>Symbol; OMIM #</th>
<th>Location</th>
<th>Disease Phenotype</th>
<th>Disease Protein</th>
<th>Function</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>MERTK 604705</td>
<td>2q14.1</td>
<td>Recessive RP</td>
<td>A c-mer protooncogene receptor tyrosine kinase</td>
<td>Involved in the phagocytosis of photoreceptor outer segments</td>
<td>D'Cruz et al, 2000; Gal et al, 2000,</td>
</tr>
<tr>
<td>PROM1</td>
<td>4P15.32</td>
<td>Recessive retinal degeneration</td>
<td>Prominin (mouse) like 1</td>
<td>A 5-transmembrane glycoprotein associated with plasma membrane evaginations in rod outer segments</td>
<td>Maw et al, 2000</td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symbol; OMIM #</td>
<td>Location</td>
<td>Disease Phenotype</td>
<td>Disease Protein</td>
<td>Function</td>
<td>References</td>
</tr>
<tr>
<td>IMPDH1 146690</td>
<td>7q31.1</td>
<td>Dominant RP</td>
<td>Inosine monophosphate dehydrogenase</td>
<td>Catalyzes the rate-limiting step in de novo guanine synthesis</td>
<td>Bowne et al, 2002; Bowne et al, 2006</td>
</tr>
<tr>
<td>RLBP1 180090</td>
<td>15q26.1</td>
<td>Recessive RP</td>
<td>Cellular retinaldehyde binding protein</td>
<td></td>
<td>Eichers et al, 2002</td>
</tr>
<tr>
<td>UNC119 (HGR4) 604011</td>
<td>17q11.2</td>
<td>Dominant cone-rod dystrophy</td>
<td>Human homolog of C. elegans unc119 protein</td>
<td>A photoreceptor synaptic protein localizes to rod and cone ribbon synapse</td>
<td>Kobayashi et al, 2000</td>
</tr>
<tr>
<td>CERKL 608380</td>
<td>2q31.3</td>
<td>Recessive RP</td>
<td>Ceramide kinase-like protein</td>
<td>Ceramide kinase proteins are involved in neuronal cell</td>
<td>Tuson et al, 2004</td>
</tr>
<tr>
<td>Locus</td>
<td>Chromosome</td>
<td>Phenotype</td>
<td>Gene Function</td>
<td>Protein Localization</td>
<td>Reference</td>
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<tr>
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<td>---------------</td>
<td>----------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RIMS1 603649</td>
<td>6q13</td>
<td>Dominant cone-rod dystrophy</td>
<td>Regulating synaptic membrane exocytosis protein 1</td>
<td>Protein localizes to ribbon synapses and interacts with RAB3A, a protein that regulates synaptic vesicle exocytosis</td>
<td>Johnson et al, 2003</td>
</tr>
<tr>
<td>CA4 600852</td>
<td>17q23.2</td>
<td>Dominant RP</td>
<td>Carbonic anhydrase IV</td>
<td>Localized in retinal choriocapillaries is a zinc-containing enzyme that catalyze hydration of carbon dioxide</td>
<td>Rebello et al, 2004</td>
</tr>
</tbody>
</table>
Figure 1.3: Diagrammatic representation of some of the important genes, their locations in the photoreceptor and involvement in the phototransduction cascade.
Adapted and modified from Farrar et al. 2002
1.5.1 GENES INVOLVED IN PHOTOTRANSDUCTION CASCADE:

Rhodopsin, the rod photopigment is the most exclusively studied protein that is involved in the phototransduction and account for more than 70% of the total outer segment proteins. Mutations in the rhodopsin gene cause retinitis pigmentosa and congenital stationary night blindness. More than 100 distinct mutations have been identified that are responsible for 25% of the RP cases (Sohocki et al, 2001; Farrar et al, 2002). Mutations in rhodopsin can be classified according to their biochemical and cellular properties, which affect the processing, translocation or degeneration of wild type rhodopsin (Mendes et al, 2005). Mutant rhodopsin reportedly causes continuous activation of the phototransduction cascade that leads to photoreceptor degeneration (Iakhine et al, 2004; Chuang et al, 2004). Mislocalization of mutant rhodopsin within the photoreceptors triggers unknown pathways that subsequently cause apoptosis (Alfinito and Townes-Anderson, 2002).

GUCY2D is another gene involved in phototransduction pathway. This gene codes for an enzyme retinal guanylate cyclase that is responsible for the regeneration of the cyclic GMP during the restoration of dark-adapted state of the photoreceptor cells. Mutations in the GUCY2D gene cause severe LCA, characterized by non-recordable visual fields and stationary congenital blindness (Perrault et al, 1999). However, some cases have been reported in which mutations cause dominant CRD, a somewhat milder phenotype than LCA (Kelsell et al, 1998; Payne et al, 2001; Udar et al, 2003). It was observed that mutations in the GUCY2D severely affect retinal morphology since the patient had
a reduced number of ganglion cells and complete absence of the outer segments with preserved inner segments (Milam et al, 2003).

1.5.2 GENES INVOLVED IN VISUAL CYCLE:

RPE65 expressed in retinal pigment epithelium and plays a key role in retinoid cycle that is necessary for the regeneration of 11-cis-retinal from all-trans-retinal, a component of the visual pigment. In the absence of RPE65 function, all-trans-retinyl esters fail to convert into 11-cis-retinal and therefore accumulate in the RPE (van Hooser et al, 2000). As a result, the photoreceptor cells lack the photopigment and this ultimately causes blindness. It was also shown that all-trans-retinyl ester was accumulated in lipid like droplets in the RPE cells (Redmond et al, 1998; vanHooser et al, 2000; Seeliger et al, 2001). Mutations in the RPE65 gene are responsible for about 12% of all the LCA cases (Lorenz et al, 2000; Thompson et al, 2000). Studies on transgenic mice (Rpe65-/-) showed changes in retinal morphology that were similar to the human LCA phenotype. ERGs of Rpe65-/- mice were severely attenuated showing the disruption of both rod and cone function.

1.5.3 PHOTORECEPTOR STRUCTURAL GENES:

Rp1 protein is a structural protein known to have a crucial role in photoreceptor outersegment (OS) morphogenesis (Gao et al, 2002). Its expression is restricted to the axoneme in the connecting cilia of the outer and inner segments of photoreceptors (Liu et al, 2002). The connecting cilium contains a microtubule-based axoneme which begins at the basal body in the distal portion of the inner segment, passes through the connecting cilium, and continues into the outer segment for half of its length (Rohlich, 1975; Kaplan et
al, 1987). The outer segment discs line up along the axoneme, it has been suggested that the axoneme may stabilize the stack of disc membranes (Kaplan et al, 1987). The N-terminal region of the RP1 gene has sequence homology to the microtubule-binding domain of the doublecortin (DCX) protein (Pierce et al, 1999). Doublecortin are classical microtubule associated proteins (MAP) that are thought to mediate signal transduction pathways by assisting in microtubule polymerization and interact with JNK (c-Jun N-terminal kinase) scaffold protein. RP1 is reported to be a member of doublecortin (DCX) protein superfamily. (Reiner et al, 2006). The localization of the RP1 gene in the axoneme and the presence of DCX domain suggest its role as microtubule associated protein in controlling the outer segment organization (Liu et al, 2004).

Studies in mice with targeted disruption of the Rp1 gene suggest the role of Rp1 gene in the organization of the outer segment discs (Gao et al, 2002; Liu et al, 2003). Mice lacking Rp1 gene were observed to show rapid retinal degeneration characterized by the accumulation of small packets of intact but incorrectly oriented discs in place of outer segments. Majority of the mutations reported in the human RP1 gene are protein truncation mutations, mostly clustered in a small region of exon 4 spanning 442bp from nucleotide 1974-2345 (Guillonneau et al, 1999; Payne et al, 2000).

Retinitis pigmentosa GTPase interacting protein (RPGRIPI1) is expressed in the amacrine cells of the retina and colocalizes with RPGR protein to the connecting cilium of inner and outer segments of the photoreceptors. RPGRIPI1 protein consists of a coiled-coil (CC) domain (homologous to proteins involved in vesicular trafficking) and a C-terminal RPGR interacting domain (RID) which
interacts with the RPGR protein, providing anchorage to the RPGR in the connecting cilium and participate in the protein trafficking through its CC domain and/or disc morphogenesis (Zhao et al, 2003). Mutations in RPGRIP1 gene had been known to cause LCA (Dryja et al, 2001; Gerber et al, 2001) and autosomal recessive CRD (Hameed et al, 2003).

Peripherin 2 (retinal degeneration slow (RDS) is one of the earliest proteins to be associated with arRP, adRP and cone-rod dystrophy (Farrar, 1991; Kajiwara et al, 1991). It is a transmembrane glycol-protein and thought to establish complexes with integral role in the morphogenesis and structure of OS discs in both rods and cones. Functional RDS protein form homodimers that subsequently establish tetramers either with other peripherin homodimers or with the homodimers of a homologous protein ROM1 (rod outer segment protein 1). Simultaneous mutations in both the proteins manifest digenic RP (Kajiwara et al, 1994). Two different disease mechanisms are described in the experiments on transgenic frog and mice. First is that the mutations in the RDS protein prevent the protein to form tetramers, thus inhibiting its transport from the IS to the OS resulting in the retinal degeneration. In another mechanism, the mutant protein is incorporated into the discs but cause them to become inherently unstable and RP develops because of the degeneration of the OS (McNally et al, 2002; Loewen et al, 2003).

Another structural protein shown to cause RP is retinal fascin (FSCN2) associated with the connecting cilium (Wada et al, 2001). It is supposed to play a role in discs morphogenesis by assembling the actin-based structures in the plasma membrane of connecting cilium (Saishin et al, 2000).
1.5.4 **RETINAL TRANSCRIPTION FACTOR GENES:**

Cone-rod homeobox containing gene (*CRX*) is an important retinal transcription factor that control the development and differentiation of the photoreceptors by controlling the transcription of several retina specific genes (Furukawa *et al*, 1997). It has been suggested that mutant *CRX* causes impaired regulation of *CRX* mediated transcription within the photoreceptors (Chen, 2002). Mutations in *CRX* are responsible for causing CRD and LCA (*Freund et al*, 1997; Schorki *et al*, 1998). Both recessive and dominant mutations have been reported in this gene (*Freund et al*, 1998; Swaroop *et al*, 1999).

*NR2E3* genes present on chromosome 15q23, is a ligand-dependent transcription factor known to control many important retinal genes (*Sharon et al*, 2003).

Another important transcriptional factor gene is neural retina leucine zipper gene (*NRL*) that is involved in the development and maintenance of the photoreceptors (*Bessant et al*, 1999; Mears *et al*, 2001).

1.5.5 **GENES INVOLVED IN PHOTORECEPTOR DEVELOPMENT AND MORPHOGENESIS:**

*AIPL1* gene associated with LCA has important albeit as yet unknown roles in the differentiation and the development of the photoreceptor cell system. *AIPL1* protein is expressed in the developing cone and rod photoreceptor precursor cells. However, in the adult retina the expression of *AIPL1* protein was restricted only to the rod photoreceptor cells suggesting its role in the viability and maintenance of rod cell in the adult retina (*van der Spuy et al*, 2002; 2003; 2005). Two models for *AIPL1* function has been proposed which help in
understanding the exact role of this protein in the morphogenesis of photoreceptor cells. First is the role of AIPL1 in fernesylation reaction. It is the addition of fernesyl residue to specific proteins. AIPL1 enhances the fernesylation of retinal proteins that is necessary for the maintenance of retinal cytoarchitecture and photoreceptor structure (Ramamurthy et al, 2003). Another experiment with yeast two-hybrid system showed a potential AIPL1 interacting protein. This protein NUB1 (NEDD8 ultimate buster 1) is thought to play an important role in the regulation of cell cycle (Akey et al, 2002; van der Spuy and Cheetham, 2004). Through this interaction a role of AIPL1 in cytosolic stability and/or nuclear transport of NUB1 during the photoreceptor development has been suggested (Akey et al, 2002).

Mouse models of LCA have been engineered to study the retinal morphology and biochemistry. Aipl1-/- mice have an extinguished electroretinogram from the early stages of development. Retinal morphology appeared to be normal during development but soon after birth photoreceptor cells were observed to degenerate in the outer nuclear layer (Dyer et al, 2004; Ramamurthy et al, 2004). Similarly, another hypomorphic (h) mutant mouse had shown normal morphological retinal development with significantly reduced Aipl1 expression and this caused the degeneration of more than half of the photoreceptors at the age of eight months (Liu et al, 2004).

The gene for human SEMA4A encodes a transmembrane protein comprising 760 amino acids. SEMA4A contains a signal peptide preceding a conserved semaphorin domain (aa 64-478), followed by a PSI domain (aa 496-580), an Ig-like domain (aa 570-630), a transmembrane domain (aa680-702),
and a short (AA 703-760) cytoplasmic tail. SEMA4A is expressed in high levels in the developing and adult brain and eye. The expression of SEMA4A in the RPE and inner retinal neurons during embryonic development, at the time when RPE contacted with developing photoreceptor outer segment suggesting its role in the photoreceptor development (Rice et al, 2004).

Mouse model has shown that mutations in semaphorin4A gene severely disrupt the development of both the rod and cone photoreceptors. Depigmentation of the retinal pigment epithelium and attenuation of retinal blood vessels was evident in the retinas of mouse lacking semaphorin4A. The disruption also affects the physiological function of both rod and cone photoreceptor cells. Hence, it had been suggested that Sema4A functions as a transmembrane ligand for a receptor present on photoreceptor cells (Rice et al, 2004).

It has been observed in the mouse model lacking sema4A that initiation of the photoreceptor development was normal but they did not develop into long organized stacks of discs. It was thus suggested that semaphorin4A provides cell-cell communication between the RPE and developing photoreceptor system and transduce an organizational signal for developing photoreceptor outer segments (Rice et al, 2004).

CRB1 is the human homolog of the Drosophila transmembrane protein Crumbs (den Hollander et al, 1999). CRB1 localizes to the photoreceptor apical plasma membrane and the inner segment of mammalian photoreceptors (Pellikka et al, 2002). Mutations in the human CRB1 gene have been reported to
be responsible for 10-13% of all LCA cases (den Hollander *et al*, 2001; Lottery *et al*, 2001a; 2001b; Hanein *et al*, 2004; Booij *et al*, 2005).

*TULP1* is a member of a small family, the tubby like proteins (TULPs), all members share 63% - 90% identity in their highly conserved C-terminal region (North *et al*, 1997; Lewis *et al*, 1999; Paloma *et al*, 2000). *TULP1* gene is located on chromosome 6 and is expressed mainly in the eye and brain tissues. Mutations in the *TULP1* gene have known to cause severe retinal degenerations (Banerjee *et al*, 1998; Hagstrom *et al*, 1998). Cellular functions of tubby like genes are not fully understood. However, it has been suggested that TULPs have roles in protein trafficking in the photoreceptors development (Hagstrom *et al*, 1999, 2001; Xi *et al*, 2005), in mediation of insulin signaling (Kapeller *et al*, 1999) and in gene transcription (Boggon *et al*, 1999)

### 1.6 MOUSE MODEL FOR RETINAL DEGENERATIONS:

Animal models for human disorders have been very useful in understanding the disease aetiology. For human retinal degenerations, mouse models are one of the few systems that have been used extensively for decades (Dalke and Graw, 2005). For vision research, Keeler’s rodless mouse (*r*) was the first model carrying the mutation in the *Pde6b* gene (Keeler, 1924). This same mutation was later found in the retinal degeneration (*rd*) mouse model (Pittler and Baehr, 1991) that has been most extensively studied for human RP.

Dysfunction of the gene for rhodopsin is an important cause of RP. Mouse model lacking rhodopsin protein unable to develop rod outer segment, while it’s over expression also cause photoreceptor degeneration (Tan *et al*,...
2001). Crumbs mutations were identified in Drosophila (Tepass et al, 1990) and CRB1 is the Human homolog of Crumbs responsible for causing retinitis pigmentosa as well as LCA (den Hollander et al, 2001; Lotery et al, 2001b; Hanein et al, 2004; Booij et al, 2005). Mouse Crb1 model were reported to have degeneration of the neuronal retina during development (van de Pavert et al, 2004). Mouse models carrying Rpgr and Rpgrip1 mutations were also shown to have morphological changes in the photoreceptor outersegment (Zhao et al, 2003; Hong et al, 2004). Rpe65 protein is an important player in the recovery of photopigment in visual cycle and its dysfunction is thought to result in the accumulation of retinal esters in the retina. Studies with Rpe65 knockout mouse highlighted photoreceptor degeneration induced by the unliganded opsin in the absence of Rpe65 function (Woodruff et al, 2003). These were just a few of that mouse model that have been generated. Keeping in mind the extent of clinical and genetic heterogeneity, many more mice and other model organisms need to be produced in order to fully understand the disease mechanism.
1.7 REFERENCES:


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Hong DH, Yue G, Adamian M, Li T. (2001). Retinitis pigmentosa GTPase regulator (RPGR)-interacting protein is stably associated with the photoreceptor


CHAPTER 2

MATERIALS AND METHODS:

All the methods used for work on this thesis are described in standard manuals such as Sambrook and Russell (2001). The methods are therefore described briefly to account for any modifications that are in vogue in this laboratory.

2.1 ISOLATION OF PERIPHERAL BLOOD LYMPHOCYTES:

Blood samples were collected from all members of the families with informed consent in acid citrate dextrose (ACD) vacutainer tubes (Beckton Dickinson). It was observed in our laboratory that heparin should not be used as an anticoagulant as it subsequently interferes with the polymerase chain reaction (PCR). The blood was gently layered over 3ml Histopaque (Sigma) in a 15ml Falcon tube (Boyum, 1968). The tube was centrifuged at 400xg for 20 minutes using a benchtop (IEC HN SII) centrifuge and lymphocytes were collected from the interphase of the plasma and Histopaque. The cells were washed in wash medium containing 1% FCS (fetal calf serum) and 1% 2mM L-glutamine, 1mM sodium pyruvate, 501U/ml penicillin-G, 50μg/ml streptomycin (GPPS). The tube was centrifuged again at 300xg for 10 minutes. The supernatant was discarded, the cell pellet was washed twice with wash medium and finally resuspended in 2ml of RPMI 1640 culture medium, pH 7.2, containing GPPS and 10% FCS.

The total cell count was taken microscopically using a haemocytometer according to the manufacturer's instructions. Cell viability was measured by the trypan blue exclusion test as described by Kruse and Patterson (1973). Live cells could be seen as translucent while dead cells stained blue.
2.2 PREPARATION OF EPSTEIN-BARR VIRUS (EBV) SUPERNATANT:

Human peripheral blood lymphocytes were transformed with EBV in order to obtain an inexhaustible supply of DNA. To prepare EBV containing supernatant, B95-8 marmoset cells (1-5 x 10^6) were grown in RPMI 1640 culture medium containing GPPS and 10% FCS in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were allowed to grow to a density of approximately 5 x 10^6/ml. The flask was then placed at 34°C to allow viral growth for a week. The cells were centrifuged at 300xg for 10 minutes and filtered through a sterile 0.45μm membrane filter (Millipore). The filtrate was aliquoted in 1ml cryovials for storage at -70°C (Nilsson, 1976).

2.3 TRANSFORMATION OF LYMPHOCYTES WITH EBV:

Approximately 5 x 10^6 cells/ml were transferred to a 25cm² Falcon culture flasks containing 10ml transformation medium (RPMI1640 supplemented with GPPS, 10% FCS, 5x10⁻⁹ M 2-mercaptoethanol and 0.5μg/ml cyclosporin A (Walls and Crawford, 1987). EBV immortalized lymphoblastoid cells could be seen as characteristic free floating clumps after 1-4 weeks of culture and an additional sign of the establishment of the lymphoblastoid cell lines (LCL) was yellowing of the medium. The cultures were periodically examined and the medium was changed as required. Approximately 5x10⁷ – 10⁸ cells/tube were used for cryopreservation. All LCLs were at least in duplicates.

2.4 CRYOPRESERVATION OF LYMPHOCYTES:

After ascertaining that the viability of the transformed cells was greater than 90%, 5x10^6 viable cells were pelleted by centrifugation at 300xg for 10 minutes. The supernatant was aspirated and the pellet was resuspended in 1ml
aliquots in a freezing mixture consisting of 45% RPMI 1640, 45% FCS and 10% dimethyl sulfoxide (DMSO). The cryovials were kept overnight in a styro-foam box at -20°C and transferred the next morning into a -70°C freezer or the vapour phase of a liquid nitrogen inventory system (Taylor Wharton) (Walls and Crawford, 1987).

When required for re-growth, the frozen cells were quickly transferred into a 15ml Falcon tube containing 10ml culture medium at room temperature. Cells were centrifuged and washed twice as described above. They were then grown in culture medium according to the standard procedure.

2.5 PURIFICATION OF DNA FROM LYMPHOCYTES AND LCLs:

The method described by Maniatis et al (1982) was used to purify the DNA from lymphocytes and from LCLs with slight modifications.

Lymphocytes obtained from 20 ml blood or from the EBV transformed cells were mixed with 19ml of STE buffer (NaCl, Tris-EDTA containing 3M NaCl, 1M Tris pH 8.0 and 0.5M EDTA pH 8.0) in a sterile 50ml Falcon tube. With gentle vortexing, 10% SDS (sodium dodecyl sulphate) was added drop wise followed by 20μl of 20mg/ml proteinase K. The tube was incubated overnight in a rotary water bath at 55°C. The next day, equal volumes of Tris-equilibrated phenol (pH 8.0) was added, mixed gently for 10 minutes and kept on ice for 10 minutes. After centrifugation at 3200rpm in an MSE 3000i (Mistral) centrifuge for 40 minutes at 4°C, the aqueous layer was carefully removed with the help of cut-off 1ml micropipette tips (which had a 0.3cm diameter at the orifice). The samples were then extracted a second time by adding equal volumes of chloroform-isoamyl alcohol (24:1 v/v). The sample was mixed gently for 10 minutes, placed on ice for
10 minutes and then centrifuged at 3200rpm for 40 minutes at 4°C. The aqueous layer was again collected in another tube. Nucleic acid (DNA) was precipitated by adding one tenth volume of 10M ammonium acetate followed by two volumes of absolute ethanol (or an equal volume of isopropanol) and stored at -20°C overnight. Precipitated DNA was pelleted the next day by centrifugation at 3200rpm for 90 minutes at 4°C. The pellet was then washed with 70% ethanol and centrifuged again at 3200 rpm for 40 minutes. The pellet was vacuum dried for 10 minutes. To resuspend the DNA, 1ml of Tris-EDTA (pH 8.0) was added to each tube and kept at 37°C in a shaking water bath. After 1 hour, 10μl of RNase (10mg/ml) was added and incubated at 37°C for a further 2 hours. Then, 50μl of 10% SDS and 5μl proteinase K was added to the sample and incubated in a shaking water bath at 55°C. After the incubation, 4ml Tris-EDTA buffer (pH 8.0) was added and the sample was again extracted first with 7ml of equilibrated phenol followed by 7ml of chloroform/isoamyl alcohol (24:1). The upper aqueous layer was collected and one-tenth volume 10M ammonium acetate was added. DNA was precipitated with 2 volumes of absolute ethanol (or an equal volume of isopropanol). The precipitated DNA was kept overnight at -20°C and was pelleted by centrifugation the following day for 90 minutes at 4°C. Then 5ml of 70% ethanol was added to the DNA pellet and centrifuged again at 3200rpm for 40 minutes. The pellet was vacuum dried for 10 minutes. DNA was resuspended in 1ml of 10mM Tris (pH 8.0). The optical density (OD) was measured at 260nm and 280nm using a Hitachi U3210 spectrophotometer.

The concentration of DNA in the sample was calculated by the following formula:
Absorbance at 260nm X dilution factor X 50 = μg/ml DNA

(Where 50 is the correction factor for double stranded DNA).

If the ratio $\text{OD}_{260}/\text{OD}_{280}$ was found to be 1.7-2.0, DNA was considered pure and free of contaminating phenol or protein for further analysis.

The sample was then transferred to an appropriately labelled Eppendorf tube and stored at 4°C.

The procedure for the extraction of the DNA directly from blood is same as above with some minor modifications that are as follows. The blood was mixed with thrice the volume of cell lysis buffer (0.15M ammonium chloride, 0.01M potassium bicarbonate and 0.5M EDTA pH 8.0) and kept on ice for 30 minutes. The samples were centrifuged for 10 minutes at 1200rpm. The pellets were again washed with 10 ml of lysis buffer, centrifuged for 10 minutes at 1200rpm. To the pellet 4.75ml of STE buffer was added. With gentle vortexing, 250μl of 10% SDS (sodium dodecyl sulphate) was added drop wise followed by 10μl of 20mg/ml proteinase K. The tube was incubated overnight in a rotary water bath at 55°C. The next day, the samples were extracted using phenol and chloroform/isoamyl alcohol as mentioned earlier. After first extraction, 10μl of RNAsae (10mg/ml) was added and the samples were incubated at 37°C for 2 hours. After 2 hours 250μl of 10% SDS was added dropwise followed by 10μl of proteinase K and again incubated at 55°C for 1 hour. Step of second extraction and precipitation were same as mentioned for the lymphocytes DNA preparation.

DNA extraction was occasionally also carried out using the Nucleon DNA extraction kit (Scotlab, UK) according to the manufacturer’s instructions.
2.6 POLYMERASE CHAIN REACTION (PCR):

The discovery of the polymerase chain reaction by Saiki et al (1985) has been one of the greatest achievements that have revolutionised DNA technology. Unless stated otherwise, the conditions used are described briefly below:

PCR reactions were carried out using a master mix that is used routinely in our laboratory:

<table>
<thead>
<tr>
<th>Master mix</th>
<th>Final conc.</th>
<th>Required vol.per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionised H₂O</td>
<td></td>
<td>4.0 µl</td>
</tr>
<tr>
<td>10X PCR Buffer</td>
<td>1X</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>15 mM MgCl₂</td>
<td>1.5mM</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>2.5 mM dNTPs</td>
<td>100 µM</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>2.5 U/µl Taq Polymerase</td>
<td>2.5 U</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>20 µM Forward Primer</td>
<td>600 nM</td>
<td>0.3 µl</td>
</tr>
<tr>
<td>20 µM Reverse Primer</td>
<td>600 nM</td>
<td>0.3 µl</td>
</tr>
</tbody>
</table>

Total 8.0 µl

For a typical experiment, the total volume was 10µl. To 2µl of DNA (40ng/µl), 8µl of the master mix was added in a PCR tube. A negative (master mix only) and a positive control (master mix + successfully amplified DNA containing target sequence) were set up for each experiment. DNA amplification was carried out using a Thermo Hybaid PxE0.2 thermal cycler as follows.
PCR Conditions

1 cycle of 95°C for 4 minutes (denaturation)
30 cycles of 95°C for 45 seconds (denaturation)
55°C for 45 seconds (primer annealing)
72°C for 1 minute (extension)
1 cycle of 72°C for 7 minutes (final extension)

A number of precautions were taken to minimize the possibility of obtaining non-specific PCR products, e.g. varying the concentration of MgCl₂ or annealing temperature etc. as described in this thesis where necessary. In some instances where required, a 'hot-start' PCR method was used that involves the addition of Taq polymerase after the first denaturation cycle.

2.7 AGAROSE GEL ELECTROPHORESIS:

Agarose Gel Electrophoresis is a popular method for the separation and purification of DNA fragments.

Depending on the size of the fragment to be analysed, a 1%-2% solution of agarose (molecular biology grade; Sigma Chem. Co) was prepared in TAE electrophoresis buffer (40mM Tris acetate, 2mM EDTA, pH 8.5). The solution was heated in a loosely stoppered bottle to dissolve the agarose in a microwave oven. After mixing the solution and cooling to about 55°C, ethidium bromide was added to the solution at a concentration of 0.5μg/ml and poured onto the casting platform of a horizontal gel electrophoresis apparatus. An appropriate gel comb was inserted at one end. The bottom tip of the comb was kept 0.5-1.0mm above
the base of the gel. After the gel had hardened, the gel comb was withdrawn. Sufficient electrophoresis buffer was added to cover the gel to a depth of approximately 1mm. Each DNA sample, in an appropriate amount of loading dye (0.25% Orange G, 20%Ficoll and 100mM EDTA) was then loaded into a well with a micro-pipettor. Appropriate DNA molecular weight markers (100bp DNA molecular weight ruler, BioRad Labs. Ranging from 100-1000bp) were included in each gel. Electrophoresis was carried out at 100 volts for 30-60 minutes. The gel was visualised and recorded using a gel documentation system (Syngene, Synoptics Co.UK).

On occasions when a particular DNA fragment was required to be isolated, the appropriate band was cut out using a sterile blade or scalpel. DNA was recovered from the agarose gel band using the QIAquick gel extraction kit (QIAGEN).

2.8 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE):

An 8% polyacrylamide gel solution was prepared by adding 50 ml of a 40% acrylamide stock solution to 25 ml of 10X TBE (Trizma base; Tris [hydroxymethyl] aminomethane 70g, 55g boric acid and 9.0g ethylene diamine tetraacetic acid (EDTA), pH 8.8.2, filtered through 0.45µM Millipore membrane filter). The volume was adjusted q.s. to 250ml with de-ionized water.

First to seal the base of the gel plates, 300µl of a 25%solution of ammonium persulphate (APS) and 300µl of TEMED (N,N,N',N'-Tetramethyl-ethylenediamine) were added to 50ml of the above gel solution. The solution was poured into the base of a BioRad SequiGen electrophoresis system (using the required spacers) and the gel was allowed to polymerise for 2-3 minutes. Then,
to 200ml of the 8% acrylamide solution, 850μl of a 25% solution of APS and 150μl of TEMED was mixed. The solution was also poured between the plates after carefully removing any air bubble and a 68 wells comb (1mm thick) was inserted. The gel was allowed to polymerize for at least 2 hours before use.

2L of 1X TBE buffer was added to the upper and lower buffer chambers of the gel unit. The gel was then pre run for 10-15 minutes at 100 watts constant power (BioRad Power Pac 3000). The comb was then carefully removed and the wells were carefully washed with 1X TBE buffer. DNA samples were prepared for electrophoresis by adding 5μl loading dye (30% Ficoll, 0.25% bromophenol blue and 0.25% Xylene Cyanol) to each sample. A molecular weight marker (100bp DNA molecular weight ruler, BioRad Labs) was included in the first lane of each gel slab and 8μl of the DNA samples were carefully loaded in the appropriate wells. The gels were run at 100 watts for 4 hours or as described for a particular experiment. The gels were cut according to the expected product sizes and stained with ethidium bromide for a few minutes. After staining the gel images were recorded using Syngene Gel Documentation System.

2.9 AUTOMATED FLUORESCENT DNA SEQUENCING:

Automated sequencing (di-deoxy terminator cycle sequencing) was carried out using an ABI 377 DNA Sequencer (Perkin Elmer) and the dye terminator cycle sequencing ready reaction kit (version 3.1).

DNA was amplified by polymerase chain reaction in a 50μl reaction volume. The reaction mixture was as described in the table below:
<table>
<thead>
<tr>
<th>Reaction component</th>
<th>µl per reaction</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile deionised H₂O</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>10 x PCR buffer + 15mM MgCl₂</td>
<td>5.0</td>
<td>1X</td>
</tr>
<tr>
<td>2.5 mM dNTPs</td>
<td>2.0</td>
<td>100µM</td>
</tr>
<tr>
<td>DNA Taq polymerase</td>
<td>1.0</td>
<td>2.5 units</td>
</tr>
<tr>
<td>Primer forward (20µM)</td>
<td>1.0</td>
<td>1.0µM</td>
</tr>
<tr>
<td>Primer reverse (20µM)</td>
<td>1.0</td>
<td>1.0µM</td>
</tr>
<tr>
<td>DNA template (10ng/µl)</td>
<td>5.0</td>
<td>50ng</td>
</tr>
</tbody>
</table>

The following PCR cycling conditions were used for the amplification:

1 cycle 95°C for 4 minutes (denaturation)
35 cycles of 95°C for 1 minute (denaturation)
      55°C for 1 minute (annealing)
      72°C for 1 minute (extension)
1 cycle 72°C for 10 minute (final extension)

Amplified PCR products that were first checked on an agarose gel were precipitated with 2.5 volumes of 95% ethanol. Samples were then washed with 70% ethanol and the pellets were resuspended in autoclaved deionised water. If required, PCR products were also purified with the QIAquick PCR product extraction kit (Qiagen) according to the manufacturer's instruction.
PCR for the sequencing reaction consisted of the following:

Sterile deionised H₂O 2.0 µl

Terminator ready reaction mix.
(Includes labelled dye terminators, buffer, and dNTPs) 4.0 µl

Forward or Reverse sequence specific primer 1.0 µl

Template DNA (0.5µg) 3.0 µl

Total reaction volume 10.0 µl

PCR was performed using a Thermo Hybaid multi-block system (MBS 0.2S), or Thermo Hybaid PxE 0.2 thermal cycler for 25 cycles as follows:

96°C for 10 seconds

50°C for 5 seconds

60°C for 4 minutes

After amplification, the products were precipitated with 50ml of 95% ethanol, washed with 70% ethanol and vacuum dried. The pellets were resuspended in 5µl of ABI loading buffer, diluted with formamide (1:5), denatured at 95°C for 2 minutes and then loaded onto the sequencing gel.

2.10 PREPARATION OF SEQUENCING GEL:

To prepare sequencing gel, 9g of urea (6M) was dissolved in approximately 10ml of deionised water, placed on a hot plate with constant stirring. After dissolving the urea, 2.5ml of a 19:1 acrylamide gel solution (Sequa gel) and 2.5ml of 10X TBE was added q.s. to 25ml with sterile deionised water. The solution was filtered through a 0.2µm Millipore membrane filter and degassed using a Millipore vacuum filtration assembly. To the filtered solution,
200μl of 10% APS and 5μl of TEMED was added and immediately poured into the gel plates. The flat edge of the shark-tooth comb was inserted into the gel plates. The gel was allowed to polymerise for 30 minutes. After polymerization the comb was carefully removed and the plates were washed thoroughly with deionised water to remove any gel particle stuck to the outside of the gel plates. Plates were allowed to dry for 30 minutes, and then placed in the sequencer with a shark-tooth comb inserted. 1200 mls of 1X TBE buffer was poured in the upper and lower buffer chambers and the run was set up according to the manufacturer's instructions.

2.11 DENATURING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (DHPLC):

Denaturing high performance liquid chromatography was initially described by Oefner and Underhill (1995). When a single-copy region of genomic DNA of a heterozygous individual is amplified by PCR, its melting properties are different to that of the PCR product of the same region of DNA from a perfectly matched homozygous individual. Detection of single-base substitutions, small insertions or deletions by DHPLC exploits different rates of retention of double stranded homo- and heteroduplex species under conditions of partial thermal denaturation on a specialised reversed phase DNAsep column™ (Underhill et al 1997). DHPLC detects all combinations of nucleotide mismatches, independent of the location of the mismatch within the fragment. It is a highly sensitive and rapid analysis system that can detect nearly 100% of all mismatches.

The Transgenomic WAVE™ DNA fragment analysis system was used for DHPLC work in our laboratory. PCR for the experiment was carried out in 15 μl
total reaction volume. PCR reaction volumes and their concentrations are described below.

<table>
<thead>
<tr>
<th>Reaction component</th>
<th>µl per reaction</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile deionised H₂O</td>
<td>6.35</td>
<td></td>
</tr>
<tr>
<td>10 X PCR Buffer</td>
<td>1.5</td>
<td>1X</td>
</tr>
<tr>
<td>50mM MgCl₂</td>
<td>0.45</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>2.0 mM dNTPs</td>
<td>1.5</td>
<td>200µM</td>
</tr>
<tr>
<td>BioTaq DNA polymerase</td>
<td>0.2</td>
<td>1 unit</td>
</tr>
<tr>
<td>Primer forward (10µM)</td>
<td>1.5</td>
<td>1.0µM</td>
</tr>
<tr>
<td>Primer reverse (10µM)</td>
<td>1.5</td>
<td>1.0µM</td>
</tr>
<tr>
<td>DNA template (20ng/µl)</td>
<td>2.0</td>
<td>40ng</td>
</tr>
</tbody>
</table>

PCR cycling parameters were the same as described in the PCR section. 5 µl of each PCR product was first checked on a 2% agarose gel to analyse the quality of the product prior to DHPLC analysis. Equal volumes of the PCR products of a wild type and each (unknown/patient) sample were separately mixed and denatured at 95°C for 5 minutes. They were then allowed to reanneal by decreasing the temperature at the rate of 1.5°C/min from 95°C-25°C.

Before setting up the experiment, the instrument was initially allowed to run (purged) with 33% of buffer A (0.1M triethylammonium acetate (TEAA) solution, pH 7.0), 33% of buffer B (0.1M TEAA solution containing 25% acetonitrile, pH 7.0) and 34% of buffer C (75% acetonitrile solution) for 2-5 minutes. After purging, the column was equilibrated for 30 minutes with 50% of
buffer A and 50% buffer B at a flow rate of 0.9ml/min. Five needle and injection port washes were carried out using buffer D (8% acetonitrile).

The DNA sequence to be screened for mutations/polymorphisms was copied to the WaveMaker (version 4.1) software and the appropriate temperature and gradient method for that particular sequence was determined. A sample sheet specifying the tube numbers, injection volumes, sample IDs and gradient was prepared. The system was initialized and run according to the manufacturer's instructions.

The optimal melting temperature for any DNA fragment can be determined by electronic submission of sequence to the web site (http://insertion.stanford.edu/melt.html).

2.12 SINGLE-STRAND CONFORMATION POLYMORPHISM (SSCP):

Since a single nucleotide change in a DNA fragment can change its conformation compared with the wild type, single-strand conformation polymorphism (SSCP) has been widely used for mutation detection. When a DNA fragment carrying a change in a heterozygous condition is amplified using PCR, denatured and immediately cooled on ice, different fragments acquire different conformations and thus resolve differently on a non-denaturing polyacrylamide gel (Bosari et al, 1995)

PCR reagents and cycling parameters for SSCP analysis were used according to the standard protocols as described in the PCR section.

A 12% resolving gel was prepared by adding 12ml of a 30% polyacrylamide stock solution (30:1, acrylamide:bisacrylamide) to 6ml of 5 X TGB (125mM Tris pH8.0, 0.96M glycine) and the volume adjusted to 30ml with de-
ionized water. A 5% stacking gel solution was also prepared by adding 2.55ml of 30% acrylamide solution, 3ml of 10X Tris-glycine buffer and the volume was adjusted q.s. to 15ml. The solutions were degassed for 10–15 minutes to avoid bubble formation in the gel. Just before pouring the gel, 100µl of 10% APS and 30µl of TEMED were mixed in the resolving gel solution while 50µl of 10% APS and 15µl of TEMED were added in the stacking gel solution.

A 16cm X 14cm gel plate was used for SSCP. Thoroughly washed plates were cleaned with 70% ethanol. Plates were set using 1mm thick spacers and sealing gaskets. The gel solution was poured between the plates, leaving a space of 1-1.5 inches at the top. Deionised water was layered on the top immediately after pouring the gel. The gel was allowed to polymerise for ~1 hour. After polymerisation, water was removed by inverting the gel plates and a 1mm thick comb was inserted between the plates. The stacking gel was then poured with the help of a syringe. After 1 hour of polymerisation, the comb and sealing gaskets were removed and the plates were adjusted in the electrophoresis apparatus (Sigma-Aldrich, Techware). Tris-glycine buffer (1X), pH 8.0 was added to the upper and lower buffer chambers. To prepare the sample for loading, 10µl of the PCR product was mixed with 20µl of loading buffer (1ml, containing 750µl formamide, 3µl of 12mM NaOH, 12µl of 6mM EDTA, 10µl xylene cyanol and 10µl of bromophenol blue and deionised water). The samples were denatured at 94°C for 4 minutes and immediately placed on ice. The gels were loaded with an appropriate molecular weight marker in the first lane. Initially, the gels were run at 300V for 10-15 minutes for proper resolution and then were run at 70-80V overnight or as required depending on the product size. The next morning the
gels were treated twice in a fixative solution (0.5% acetic acid in 10% ethanol) for 3 minutes each. They were washed with 10% ethanol and stained in a staining solution (0.1% silver nitrate in 10% ethanol) for 30-60 minutes. They were washed again with 10% ethanol and developed in a developing solution (12g NaOH, 0.08g NaBHO$_4$ and 1.2ml formaldehyde in a final volume of 800ml (10%) ethanol) for 15-20 minutes. Photographs were taken under normal (white) light using the Syngene gel documentation system and saved for analysis.
REFERENCES:


CHAPTER 3

MUTATION SCREENING OF PAKISTANI FAMILIES WITH CONGENITAL EYE DISORDERS

3.1 INTRODUCTION:

Retinitis pigmentosa is both clinically and genetically a heterogeneous group of inherited ocular disorders characterized by progressive degeneration of the photoreceptors. It primarily affects the rod photoreceptors resulting in night blindness and reduction in the peripheral visual field, and progressive loss of central vision (Phelan and Bok, 2000). RP12 (MIM # 600105) is a clinically distinct and relatively severe form of autosomal recessive RP, in which there is preservation of the para-arteriolar retinal pigment epithelium (PPRPE; Heckenlively, 1982). A locus for arRP with PPRPE was mapped on chromosome 1q31 (van Soest et al, 1994; Leutelt et al, 1995). Mutations in the human homologue of the Drosophila crumbs gene (CRB1) located in this region have been reported to cause RP12 (den Hollander et al, 1999). A number of missense mutations in the CRB1 gene have also been reported for Leber congenital amaurosis (LCA) families and random patients (Lotery et al, 2001a). A few families with severe RP but without any PPRPE symptoms have also been shown with disease associated CRB1 gene mutations (Lotery et al, 2001b).

The CRB1 gene consists of 11 exons spanning at least 40kb. It is expressed specifically in the brain and retina. Human Crb1 is a transmembrane protein containing a large extracellular part. It contains three laminin G domains and nineteen epidermal growth-factor-like domain. The conserved intracellular part consists of 37 amino acids C-terminal PDZ-binding motif (Meuleman et al,
2004). The sequence and arrangement of laminin G-like domain flanked by two EGF like domains is conserved in humans and *Drosophila* gene (den Hollander *et al.*, 1999). Functions of the crumbs protein has extensively been studied in *Drosophila*, although some mouse models now been reported. In Drosophila it is localized on the apical membrane of the epithelial cells, and also in the stalk membrane apical to the zonula-adherens in the photoreceptor cells. It has been shown that *Drosophila* crumbs gene plays an important role in photoreceptor morphogenesis (Pellikka *et al.*, 2002).

Leber congenital amaurosis (LCA; MIM # 204000) is the most severe form of all inherited retinal dystrophies. Individuals affected with LCA are diagnosed with severely impaired vision or blindness at birth or within the first few months of life. Little is known about the pathophysiology of LCA and the defect is regarded as a consequence of either impaired development of photoreceptors or extremely early degeneration of cells that have developed normally (Perrault *et al.*, 1999). Like other retinal disorders it is also genetically heterogeneous. To date, several loci/genes for LCA had been reported on different chromosomes (http://www.sph.uth.tmc.edu/RetNet/). The loci for both LCA1 and LCA4 have been reported on chromosome 17p13.1. Gene mutations have also been reported in candidate genes *GUCY2D* (Perrault *et al.*, 1996) and *AIPL1* (Sohocki *et al.*, 2000a) for LCA1 and LCA4 respectively.

Many LCA families of Pakistani origin mapped on 17p13 have been shown to have *AIPL1* gene mutations. The interesting observation is that none of the 17p13.1 linked Pakistani LCA families that have been reported so far, had disease associated mutations in the *GUCY2D* gene.

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The *AIPL1* gene encodes a protein named aryl hydrocarbon receptor-interacting protein-like1. *AIPL1* consists of six exons encoding a 384 amino acids protein. It contains three tetraticopeptide motifs, indicating a possible role in nuclear transport or protein chaperone activity. However, the function of this protein in normal vision is still unknown (Ma and Whitlock, 1997).

### 3.2 MATERIALS AND METHODS:

Several consanguineous Pakistani families suffering from RP12 and LCA were ascertained for this study. The parents of the patients in all the families were unaffected indicating an autosomal recessive mode of inheritance. The RP families with PPRPE had an early onset of the disease (average 7-15 years) with the gradual deterioration of visual acuity. By the age of 25 affected individuals were completely blind. At an early age their best visual acuity was achieved with a correction of +3.5D up to +7.0D. There was a high level of pigmentation, distributed in all four quadrants of the retina. However, sparing of the para arteriolar space was quite clear at early stages of the disease (Figure 3.1). The macular degeneration was also associated with the disease process. Majority of the affected individuals had cells in the anterior vitreous and they also had sub-capsular cataract.

Clinical examinations of LCA families showed that the fundus of the patients had marked macular degeneration at early stages of life and diffuse pigmentary retinopathy later. Some affected subjects also had early signs of keratoconous. There was no other abnormality associated with the disease.
Figure 3.1:
Photograph of the fundus of a RP12 (PPRPE) patient. All four quadrants of the retina are highly pigmented. Arrows shows absence of pigmentation in a para arteriolar space.
Peripheral blood samples were collected with the informed consent of the families and used for genetic analysis. Blood samples were also collected from 100 ethnically matched unrelated normal Pakistani individuals and were used as controls for allele frequencies and mutation screening. Genomic DNA was extracted from whole blood using an extraction kit (Nucleon II; Scotlab Bioscience; Strathclyde, Scotland, UK).

3.2.1 Microsatellite Analysis:

Alleles for the arRP/LCA linked microsatellite markers were amplified from genomic DNA samples of all family members using primers for these markers (Research Genetics; V-8) (http://www.sph.uth.tmc.edu/Retnet/disease.htm). Nonradioactive polymerase chain reaction (PCR) was performed in a 10ul reaction with 300ng of genomic DNA, according to the standard protocol. The amplified products were separated by electrophoresis on 8-10% polyacrylamide gels and stained with ethidium bromide, as described in the Materials and Methods chapter.

3.2.2 Mutation Screening:

Mutation screening was carried out using heteroduplex and sequence analysis of the genomic DNA. The sequences for specific gene primers and amplification conditions used for heteroduplex analysis and mutation screening are given in table 3.1 and 3.2.

Heteroduplex analysis was performed using an automated DHPLC instrumentation (WAVE DNA fragment analysis system; Transgenomic, Crewe, UK). Sample preparation for heteroduplex analysis was carried out by denaturing
and reannealing of unpurified PCR products of carriers (heterozygotes). Temperature conditions required for successful resolution of heteroduplexes were obtained from the website (http://insertion.stanford.edu/melt.html).

Once heteroduplex peaks had been detected, all family members were sequenced in forward and reverse directions using a commercially available kit (Big Dye, Perkin Elmer, ABI) and the products were analyzed on an automated sequencer (Model 377; Perkin Elmer. ABI).

To exclude the possibility of these mutations being a polymorphism, 100 control samples, were also screened.
### TABLE 3.1:

**PRIMER PAIRS AND PCR CONDITIONS FOR MUTATION SCREENING OF THE AIP1L1 GENE:**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer Sequence (5' &gt; 3')</th>
<th>PCR product size (bp)</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1F</td>
<td>GGACACCTCCCTTTCTCC</td>
<td>244</td>
<td>69°C X 1.0mM Mg⁺²</td>
</tr>
<tr>
<td>1R</td>
<td>GCTGGGGCTGCCTGGCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2F</td>
<td>GGGCCCTTTGAACAGTGTTGTCT</td>
<td>320</td>
<td>58°C X 1.5mM Mg⁺²</td>
</tr>
<tr>
<td>2R</td>
<td>TTTCCCGAAACACAGCAGCAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3F</td>
<td>AGTGAGGGAGCAGGATTC</td>
<td>368</td>
<td>54°C X 1.5mM Mg⁺²</td>
</tr>
<tr>
<td>3R</td>
<td>TGCCCATGATGCCCGCTGTGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4F</td>
<td>TTTCCGGTCTCTGATGGG</td>
<td>349</td>
<td>62°C X 1.5mM Mg⁺²</td>
</tr>
<tr>
<td>4R</td>
<td>GCAGGCTCCCCAGAGTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5F</td>
<td>GACTCTGGGGGGGCCTGCCCCA</td>
<td>328</td>
<td>50°C X 1.5mM Mg⁺²</td>
</tr>
<tr>
<td>5R</td>
<td>GTGGGGTGGAAAGAAAAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6F</td>
<td>CTGGGAAGGGAGCTGTAG</td>
<td>501</td>
<td>67°C X 1.5mM Mg⁺²</td>
</tr>
<tr>
<td>6R</td>
<td>AAAAGTGACACCACGATCC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
# TABLE 3.2:
PRIMER PAIRS AND PCR CONDITIONS FOR MUTATION SCREENING OF THE CRB1 GENE:

<table>
<thead>
<tr>
<th>EXON #</th>
<th>FORWARD PRIMER</th>
<th>REVERSE PRIMER</th>
<th>PCR product size (bp)</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CAGCAACACACCAGAGGATG</td>
<td>ATATAACGCCCAACTAACCAG</td>
<td>158</td>
<td>60°C X 1.5mM Mg²⁺</td>
</tr>
<tr>
<td>2.1</td>
<td>GGTGAGGCAGCACAAGGCTC</td>
<td>CAGGAGTCTTGGCACCACGG</td>
<td>375</td>
<td>60°C X 1.5mM Mg²⁺</td>
</tr>
<tr>
<td>2.2</td>
<td>GTACAGTGGGACAATCTGTG</td>
<td>CCAAGTCGCACTGTCTGACC</td>
<td>254</td>
<td>62°C X 1.5mM Mg²⁺</td>
</tr>
<tr>
<td>2.3</td>
<td>GATGGGAATTGTATGGTACTCC</td>
<td>TCACCTCTGCTGGCTGCCAC</td>
<td>229</td>
<td>60°C X 1.5mM Mg²⁺</td>
</tr>
<tr>
<td>3.1</td>
<td>GCTCTGGTTAACAACAGCATG</td>
<td>GAATCCAGGGGCACAGCTG</td>
<td>200</td>
<td>60°C X 1.5mM Mg²⁺</td>
</tr>
<tr>
<td>3.2</td>
<td>GAGCAATTTGGTGCTTCCAGC</td>
<td>CAGGAAGTGAAAATAGTTGATG</td>
<td>242</td>
<td>60°C X 1.5mM Mg²⁺</td>
</tr>
<tr>
<td>4</td>
<td>GAAACAGTATATAAGATATCTGATC</td>
<td>GCTATAAGCAGATATGTGATATC</td>
<td>275</td>
<td>60°C X 3.0mM Mg²⁺</td>
</tr>
<tr>
<td>5</td>
<td>CAACGTGATAGATCGAGCTC</td>
<td>CACAGCTTCTCTGCTAAC</td>
<td>383</td>
<td>60°C X 1.5mM Mg²⁺</td>
</tr>
<tr>
<td>6.1</td>
<td>ACAAGTAAATTACGTGAAACTTC</td>
<td>AGTGAGGGATGCGATGTTCC</td>
<td>281</td>
<td>60°C X 1.5mM Mg²⁺</td>
</tr>
<tr>
<td>6.2</td>
<td>ATCTCTCTGGGCTGTACC</td>
<td>GCTATGTTACAAACTGAGCC</td>
<td>212</td>
<td>60°C X 1.5mM Mg²⁺</td>
</tr>
<tr>
<td>6.3</td>
<td>GCGATGCTGCTCTTCGGG</td>
<td>TGCCACTCTCTACGGCTGG</td>
<td>235</td>
<td>60°C X 1.5mM Mg²⁺</td>
</tr>
<tr>
<td>6.4</td>
<td>CAGGTCAATAATCGGTCAAGG</td>
<td>CAAACGAAGGTGTGGATGGC</td>
<td>279</td>
<td>60°C X 1.5mM Mg²⁺</td>
</tr>
<tr>
<td>6.5</td>
<td>ACCAGTTGGGATGACACGC</td>
<td>CTGTTGGCAKTGACACTGG</td>
<td>252</td>
<td>60°C X 1.5mM Mg²⁺</td>
</tr>
<tr>
<td>6.6</td>
<td>CAACCTTGTCAAACAGCAGAGG</td>
<td>CTCTGAGGGCATGGCACTCC</td>
<td>140</td>
<td>60°C X 1.5mM Mg²⁺</td>
</tr>
<tr>
<td>7.1</td>
<td>TTCTCCTCTCCTATATTTTG</td>
<td>ACACCGGATATTGTAAGTGC</td>
<td>196</td>
<td>60°C X 3.0mM Mg²⁺</td>
</tr>
<tr>
<td>7.2</td>
<td>CTCAATTTTCTATAGTCCG</td>
<td>TCCTGCTTGTGAGTGAGGC</td>
<td>303</td>
<td>60°C X 1.5mM Mg²⁺</td>
</tr>
<tr>
<td>7.3</td>
<td>TCACTCCTCACAACACATG</td>
<td>ATAAAGTTAAAGTGGTAGACAG</td>
<td>308</td>
<td>60°C X 1.5mM Mg²⁺</td>
</tr>
<tr>
<td>8</td>
<td>CAACATTTTCTATAGTCCG</td>
<td>CTCAATGTTGCAACCACTG</td>
<td>276</td>
<td>60°C X 1.5mM Mg²⁺</td>
</tr>
<tr>
<td>9.1</td>
<td>AATGATCATATATTATTAACAGG</td>
<td>GTGCCATCATCAGCTACTG</td>
<td>346</td>
<td>60°C X 3.0mM Mg²⁺</td>
</tr>
<tr>
<td>9.2</td>
<td>GTGGCAACACGCTTATATAGC</td>
<td>CATGAACCATTTCCAAGTAGAG</td>
<td>303</td>
<td>58°C X 1.5mM Mg²⁺</td>
</tr>
<tr>
<td>9.3</td>
<td>ATATAAAAGGGCTGCAAGGG</td>
<td>GCTGCAACTCTGTGCAGCG</td>
<td>325</td>
<td>60°C X 1.5mM Mg²⁺</td>
</tr>
<tr>
<td>9.4</td>
<td>GAACATCAACAGGGCAGATGC</td>
<td>CATGATGCAGAGTAGATGCTTC</td>
<td>282</td>
<td>60°C X 3.0mM Mg²⁺</td>
</tr>
<tr>
<td>10</td>
<td>CTTGAATGAGATGAAACAAGATG</td>
<td>GAGGAAGAGAATGATTTGAG</td>
<td>261</td>
<td>62°C X 3.0mM Mg²⁺</td>
</tr>
<tr>
<td>11.1</td>
<td>ACCAATGATTTCAACAGGGACC</td>
<td>TTCACGTCCACCTCAGCA</td>
<td>183</td>
<td>60°C X 1.5mM Mg²⁺</td>
</tr>
<tr>
<td>11.2</td>
<td>TCTGGCCAGGACCTACTC</td>
<td>CAGAGATCTAAAATGAATCA</td>
<td>279</td>
<td>56°C X 3.0mM Mg²⁺</td>
</tr>
</tbody>
</table>
3.3 RESULTS:

In order to map the disease-causing gene(s) for the arRP (PPRPE) and LCA families, genetic linkage analysis was carried out. While testing known loci for RP, significant linkage was obtained between the phenotype of three families (3330RP, 111RP and 010LCA) and markers D1S413 and D1S1660 at chromosome 1q (Zmax 3.79, θmax = 0). This chromosomal region (1q31-32.1) harbors a gene CRB1 that has previously been reported to be associated with RP12 (den Hollander et al, 1999) and LCA (Lotery et al, 2001b).

Significant linkage for another LCA family (011LCA) was obtained on chromosome 17p13.1 with microsatellite markers D17S1294 and D17S796. Two candidate genes, GUCY2D and AIPL1 are present in this region of chromosome 17. Genotypic data of all the individuals examined from these retinal dystrophy families is shown in Figures 3.2A, 3.3A, 3.4A and 3.5A.

DHPLC analysis was performed with samples from unaffected carriers of the 1q31 and 17q13.1 linked families for disease-associated mutations in candidate genes. Heteroduplex mismatches were recognized by the appearance of more than one peak in the elution profile. The presence of heteroduplex peaks in the unaffected carrier individuals was convincing enough to do sequencing to identify the exact mutational change.
3.3.1 3330RP Family:

Sequence analysis of the CRB1 gene for the family 3330RP (RP12) revealed a single base change (G to A substitution) at nucleotide position 2536 (Fig. 3.2B). This substitution replaces glycine with an arginine at codon 846 in exon 7. This homozygous change was observed in all the affected members of this family. Parents of the affected individuals were heterozygous for this change. The substitution of glycine to an arginine may alter the conformation of the protein product and hence its biological activity. This G to A mutation associated with this RP12 family is a new mutation.
Figure 3.2A: Pedigree of RP with PPRPE family (3330RP) along with their genotypes, analyzed for disease susceptibility gene.
Figure 3.2 B

Selected electropherogram of individuals V:3 (carrier; left) and VI:11 (patient; right) from 3330RP family showing a G to A transversion in exon 7 of the CRB1 gene.
3.3.2 111RP Family:

Sequence analysis of the CRB1 gene of this RP12 family showed a T to C substitution at nucleotide position 3347 in exon 9 (Fig. 3.3B). This homozygous substitution replaces leucine (TCA) with proline (CCA) at codon 1071, which may lead to a change in the orientation of the polypeptide chain.
Figure 3.3A: Pedigree of RP with PPRPE family (111RP) along with their genotypes, analyzed for disease susceptibility genes.
Figure 3.3 B
Selected electropherogram of individuals IV:2 (carrier; left) and V:1 (patient; right) from 111RP family showing a T to C substitution in exon 9 of the CRB1 gene.
3.3.3 LCA Family:

Sequencing of the PCR product of exon 9 of CRB1 gene in the LCA family revealed a T to C substitution at nucleotide position 3101/codon 989 in all the patients (Fig. 3.4B). This homozygous transversion co-segregates with the LCA phenotype and replaces the normal iso-leucine with a threonine residue. The replacement of the hydrophobic isoleucine with a polar threonine could prevent the normal folding of the molecule. This is a previously unreported mutation associated with LCA phenotype.
Figure 3.4A:
Pedigree of LCA family (010LCA) along with their genotypes, analyzed for disease susceptibility genes.
Figure 3.4 B
Selected electropherogram of individuals IV:4 (carrier; left) and V:3 (patient; right) from 010LCA family showing a T to C substitution in exon 9 of the CRB1 gene.
3.3.4 011LCA Family:

No disease-associated mutation was found in the *GUCY2D* gene for the LCA family 011LCA. Mutation screening of *AIPL1* gene revealed a novel disease associated mutation in exon 2. All the affected individuals were homozygous for a C to A transversion at nucleotide position 116 in exon 2 that alters the codon (AA39) from threonine to asparagine (Fig. 3.5B). The threonine at this position is conserved in the rat and human *AIPL1*, as well as in the human and mouse AIP.

None of these above mentioned mutations or any polymorphism in *CRBI* and *AIPL1* genes were observed in the 100 random samples obtained from normal Pakistani individuals who had no eye disease.
Figure 3.5A:
Pedigree of LCA family (011LCA) along with their genotypes, analyzed for disease susceptibility genes.
Figure 3.5 B
Selected electropherogram of individuals III:1 (carrier; left) and IV:3 (patient; right) from 011 LCA family showing a C to A transversion in exon 2 of the AIPL1 gene.
Table 3.3:
Summary of mutations identified in Pakistani RP/LCA families

<table>
<thead>
<tr>
<th>Family ID</th>
<th>Exon (gene)</th>
<th>Mutation</th>
<th>Position (nt)</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>3330RP (RP12)</td>
<td>7 (CRB1)</td>
<td>G → A</td>
<td>2536</td>
<td>Gly846Arg</td>
</tr>
<tr>
<td>111RP (RP12)</td>
<td>9 (CRB1)</td>
<td>T → C</td>
<td>3347</td>
<td>Leu1071Pro</td>
</tr>
<tr>
<td>010LCA</td>
<td>9 (CRB1)</td>
<td>T → C</td>
<td>3101</td>
<td>Leu989Thr</td>
</tr>
<tr>
<td>011LCA</td>
<td>2 (AIPL1)</td>
<td>C → A</td>
<td>116</td>
<td>Thr39Asp</td>
</tr>
</tbody>
</table>
3.4 DISCUSSION:

The disease-associated mutations identified in these Pakistani families are summarized in Table 3.3. All mutations found in \textit{CRB1} gene are in the laminin G domains of the protein (XM 010684), which indicates the functional importance of these domains. The biochemical function of the \textit{CRB1} gene and therefore its actual role in RP and LCA is still unknown. Its retinal expression and structural similarity with the proteins that are involved in neuronal development suggest that it could be involved in the development of the retina (Kallunki and Tryggvason, 1992). However, it has been suggested that Drosophila crumbs protein plays a central role in establishing apical-basal polarity in epithelial cells (Wodarz \textit{et al}, 1995) and is essential for photoreceptor morphogenesis. It has also been reported that mutations in this gene result in disorganization of ectodermally derived epithelia and eventually leads to cell death in the disrupted tissue (Tepass \textit{et al}, 1990).

Further analysis of the protein and their interacting factors will help in determining its role in the mechanisms of retinal differentiation and human \textit{CRB1}-related retinal dystrophies.

Several mutations in the LCA4 families and adRP/adCORD cases have been reported in different exons of the \textit{AIPL1} gene (Sohocki \textit{et al}, 2000a, 2000b; Damji \textit{et al}, 2001). 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 \textit{AIPL1} gene mutations in Pakistani families.
We cannot be certain that these are the disease-causing mutations without performing biochemical analysis of the mutant protein. However, as the disease-associated mutations were found to be segregating only among the patients in families giving a LOD score greater than 3, and were absent in the unaffected family members and in 100 ethnically matched control subjects, they have to be considered to be the cause of the disease.
3.5 REFERENCES:


Ma Q. and Whitlock JP Jr. (1997). A novel cytoplasmic protein that interacts with the Ah receptor, contains tetrameric peptide repeat motifs, and augments the


q32.1 in an inbred and genetically heterogeneous disease population. Genomics. 22: 499-504.

CHAPTER 4

EVIDENCE OF \textit{RPGRIP1} GENE MUTATIONS ASSOCIATED WITH

RECESSIVE CONE-ROD DYSTROPHY

4.1 INTRODUCTION:

Cone-rod dystrophies (CRD) are a form of inherited retinal dystrophy, which characteristically lead to early impairment of vision. An initial loss of color vision (cone mediated functions) and of visual acuity, usually from the 1\textsuperscript{st} or 2\textsuperscript{nd} decade of life, is followed by night blindness (largely rod mediated) and loss of peripheral visual fields (Moore, 1992). CRD patients suffer from severe photophobia and demonstrate reduced ERG responses. In later life, vision may be reduced to a bare perception of light. CRD is a milder condition compared to Leber congenital amaurosis (LCA) which is the most severe form of all the inherited retinal dystrophies and is diagnosed as bilateral congenital blindness, with a diminished or absent electroretinogram (ERG). Cone-rod dystrophy loci have been mapped to chromosomes 18q, 17q, 19q, 17p13, 6q, 1q12, 8p11 (Warburg \textit{et al}, 1991; Klystra and Aylsworth, 1993; Evans \textit{et al}, 1994; Balciuniene \textit{et al}, 1995; Kelsell \textit{et al}, 1998; Payne \textit{et al}, 1999; Khaliq \textit{et al}, 2000; Danciger \textit{et al}, 2001). Mutations in the \textit{peripherin/RDS}, CRX, \textit{RetGC-1} and \textit{GUCA1A} genes have been shown to cause autosomal dominant CRD (Nakazawa \textit{et al}, 1994; Freund \textit{et al}, 1997; Swain \textit{et al}, 1997; Kelsell \textit{et al}, 1998; Downes \textit{et al}, 2001). Mutations in the ATP binding cassette transporter rim protein (ABCR) gene have been shown to be associated with autosomal recessive CRD (Cremers \textit{et al}, 1998). Mutations in the \textit{CNAGA3} gene encoding
the α-subunit of the cone photoreceptor cGMP-gated channel have also been reported to cause cone photoreceptor disorders (Wissinger et al, 2001).

The RGPRIP1 protein (retinitis pigmentosa GTPase regulator interacting protein 1; MIM 605446) is encoded by the gene located on chromosome 14q11. It consists of 24 exons and the predicted size of its protein product is 1259 amino acids. It is expressed specifically in the rod and cone photoreceptors and is a structural component of the ciliary axoneme. One of its functions is to anchor the RGPR protein within the photoreceptor connecting cilium (Roepman et al, 2000). Recently, in an in vivo investigation for the RGPRIP1 function and its physical interaction, it has been shown that RGPRIP1 is essential for RGPR function and is also required for normal disk morphogenesis (Zhao et al, 2003). Mutations in RGPRIP1 have been reported to be a cause of LCA (Dryja et al, 2001; Gerber et al, 2001). Here we report the first observation of the involvement of RGPRIP1 gene mutations as a cause of CRD in four Pakistani families.

4.2 SUBJECTS AND METHODS:

20 members of a two-generation and 19 members of a three-generation, consanguineous Pakistani families, 1CRD and 4CRD respectively, were studied. The 1CRD family consisted of 8 affected and 12 unaffected individuals and the 4CRD consisted of 8 affected and 11 unaffected individuals (Fig.4.1A & 4.2A). An ophthalmologist clinically examined all the patients and their unaffected family members. In all the patients, deterioration in central vision and colour blindness was from an early age and there was a rapid loss of vision between the ages of 14-16 years (visual acuity 1/60, 0.01). Patients also had severe photophobia since their childhood. Fundoscopy revealed variable degree of fundus granularity
and macular degeneration. The affected individual IV:7 (age 14 years) had characteristic macular bull’s eye lesion in both eyes. Full field flash ERG was used to measure functions of both cones and rods. Both photopic and scotopic full field ERG amplitudes were reduced, demonstrating involvement of both photoreceptor systems. However, among the young patients, cone response was reduced more than that of their rod response. Based on family history and clinical diagnosis, the disease was classified as autosomal recessive cone-rod dystrophy (arCRD).

4.2.1 Linkage Analysis:

For genetic analysis peripheral blood samples were collected with informed consent from all members of the two families and from 100 ethnically matched control subjects. Genomic DNA was extracted from whole blood using the Nucleon II extraction kit according to the manufacturer’s instructions (Scotlab Bioscience). DNA was amplified using primers for polymorphic microsatellite markers (Research Genetics) specific for known loci/genes associated with various retinal degenerations. PCR was under standard conditions described in the Materials and Methods section.

4.2.2 Mutation Detection:

Exon specific intronic primers were designed from the genomic sequence of the \textit{RPGRIPI} gene (NM 020366; Table 4.1). PCR was performed in a 50ul reaction volume using 1ug of genomic DNA. The resulting product was allowed to cool slowly to room temperature to maximize the formation of heteroduplexes. Heteroduplex analysis was performed using an automated DHPLC instrumentation (WAVE DNA fragment analysis system; Transgenomic, Crewe,
Sample preparation for heteroduplex analysis was carried out by denaturing and reannealing of unpurified PCR products of the carriers (heterozygotes). The temperature conditions required for the successful resolution of heteroduplexes were obtained from the website (http://insertion.stanford.edu/melt.html). The heteroduplex mismatches were detected using the WAVE DNA fragment analysis system (Underhill et al, 2000).

On identification of heteroduplex peaks in the carriers, all family members were sequenced in the forward and reverse directions using a commercially available kit (Big Dye, ABI) and the products were analyzed on an ABI Prism 377 automated DNA sequencer. Subsequently, six small families with CRD (5, 6, 7, 8, 9 and 10CRDs) and two with LCA were also included for mutation screening of the RPGRIP1 gene. To exclude the possibility that the mutations are polymorphisms, 100 ethnically matched control samples were also screened for heteroduplexes. For the control samples, the unpurified PCR products and the homozygous wild type reference DNA sample were mixed in an equimolar ratio. The mixture was then subjected to a 3 min, 95°C denaturing step followed by gradual reannealing from 95-65°C over 30 min. for heteroduplex analysis as described in the chapter 2.
### TABLE 4.1:

PRIMER PAIRS AND PCR CONDITIONS FOR MUTATION SCREENING OF THE RPGRIPI1 GENE:

<table>
<thead>
<tr>
<th>EXON</th>
<th>PRIMER SEQUENCE (5' &gt; 3')</th>
<th>PCR product size (bp)</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1F</td>
<td>GCTCAGTCAGATATCTGAG</td>
<td>495</td>
<td>55°C X 1.5Mm Mg²</td>
</tr>
<tr>
<td>1R</td>
<td>GAATAGGATCAAGTGGAGGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2F</td>
<td>CTTGATCCAACACTCTGACC</td>
<td>429</td>
<td>55°C X 1.5Mm Mg²</td>
</tr>
<tr>
<td>2R</td>
<td>CGGAGCCCTGCACTGAGCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3F</td>
<td>GGAGTAGT TTCTGTGCTGTTG</td>
<td>354</td>
<td>56°C X 1.5Mm Mg²</td>
</tr>
<tr>
<td>3R</td>
<td>GCCCTGGAACCTAGTTAGTGCTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4F</td>
<td>CTTGACCTAGGCACTGGCACAC</td>
<td>323</td>
<td>55°C X 1.5Mm Mg²</td>
</tr>
<tr>
<td>4R</td>
<td>CACCCCTCTGACAAGTACAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.1F</td>
<td>GAATCAGTGTTTGCTTG</td>
<td>470</td>
<td>50°C X 1.5Mm Mg²</td>
</tr>
<tr>
<td>5.1R</td>
<td>GGCTAGGCTCTCTGACAGAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.2F</td>
<td>GAACCTGCACTCCACCAGG</td>
<td>382</td>
<td>55°C X 1.5Mm Mg²</td>
</tr>
<tr>
<td>5.2R</td>
<td>GGGAAATCTCAGCCTGAGTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6F</td>
<td>CACCAATGCAGATTTCCC</td>
<td>349</td>
<td>58°C X 1.5Mm Mg²</td>
</tr>
<tr>
<td>6R</td>
<td>CTGAACCTGTGCTCATGAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7F</td>
<td>CTCTTTCTCACCACAGATCC</td>
<td>522</td>
<td>58°C X 1.0Mm Mg²</td>
</tr>
<tr>
<td>7R</td>
<td>CTGTTTCAAGCAAGTAATCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8F</td>
<td>GGAAGTAGATAAGTGCTGCTG</td>
<td>382</td>
<td>55°C X 1.5Mm Mg²</td>
</tr>
<tr>
<td>8R</td>
<td>GTGCCTGGGATTACAGGCCGTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9F</td>
<td>GATGGAGCGAAAGCTGGGG</td>
<td>479</td>
<td>58°C X 1.5Mm Mg²</td>
</tr>
<tr>
<td>9R</td>
<td>GTGCTTATCTCTGGCTGCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10F</td>
<td>GAAAGCAGGAGAAGGATG</td>
<td>328</td>
<td>58°C X 1.5Mm Mg²</td>
</tr>
<tr>
<td>10R</td>
<td>CCAACATGATGAAACCCTCCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11F</td>
<td>CTCCGGAACCTAGTGACAGAC</td>
<td>302</td>
<td>60°C X 1.5Mm Mg²</td>
</tr>
<tr>
<td>11R</td>
<td>AAGTGCTGGGATTACAGGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12F</td>
<td>GAGAGTACCGTAATGGG</td>
<td>351</td>
<td>50°C X 1.5Mm Mg²</td>
</tr>
<tr>
<td>12R</td>
<td>GTAATTCTGACCCCTCAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13F</td>
<td>CTAGATCCAGGGCAAGGATG</td>
<td>292</td>
<td>60°C X 1.5Mm Mg²</td>
</tr>
<tr>
<td>13R</td>
<td>CATCAGCAACAAAAACCAACTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14F</td>
<td>GTGATTCCAGAAGACGTG</td>
<td>329</td>
<td>60°C X 1.0Mm Mg²</td>
</tr>
<tr>
<td>14R</td>
<td>TAGTAATAATTATGATATTGAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15F</td>
<td>GCAATCCCCAGTACCTAACCTG</td>
<td>271</td>
<td>60°C X 2Mm Mg²</td>
</tr>
<tr>
<td>15R</td>
<td>GCAAGCTAACTTATATAAGAGC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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4.3 RESULTS:

Exclusion studies on both the large families (1CRD and 4CRD) showed linkage with the microsatellite marker D14S1023 (Zmax = 5.17 and 4.21 for the two families respectively at θ = 0.00) at chromosome 14q11, a locus for the *RPGRIP1* gene (Fig. 4.1A & 4.2A). Mutation screening of the candidate gene, *RPGRIP1*, was carried out to identify the disease-associated mutations.

Initially, DHPLC analysis was performed with samples from unaffected carriers of the families. Heteroduplex mismatches were recognized by the appearance of more than one peak in the elution profile. The presence of heteroduplex peaks in the unaffected carrier individuals was convincing enough to do sequencing to identify the exact mutational change.

Sequence analysis of *RPGRIP1* gene for 1CRD family revealed a homozygous G-to-T point mutation in exon 16 in all the affected individuals (Fig 4.1B). This substitution at nucleotide 2480 changes codon 827 from CGC (Arg) to CTC (Leu). This change in exon 16 was not found in any other Pakistani family studied here, the 100 control samples nor any of the unaffected individuals of the 1CRD family.
**Figure 4.1A:**

Pedigree of 1CRD family with cone rod dystrophy along with their genotypes.
Figure 4.1B:
Selected electropherograms of individuals from the 1CRD family. The left electropherogram is for the heterozygous carrier (Individuals III:4) and the right electropherogram is of the patient IV:5 that is homozygous for the G-T transversion in exon 16 of the RPRIP1 gene.
In the affected members of the 4CRD family, a G-to-T substitution was found in exon 13 at nucleotide 1639 (Fig. 4.2B). This point mutation changes codon 547 from GCT (Ala) to TCT (Ser). The same mutation was found in two other small families 5CRD, and 10CRD. No disease-associated mutation was observed in the *RPGRIP1* gene sequence for the remaining CRD & LCA families examined.

In addition, 3 polymorphisms were also identified in the *RPGRIP1* gene that include a CTC-to-CTT (Leu427Leu) polymorphism in exon 16, a G-to-A sequence change in intron 9 and a deletion (9 base pair) in the intronic region of exon 13, 32bp downstream from exon 13.
Figure 4.2A:

Pedigree of 4CRD family with cone rod dystrophy along with their genotypes.
Figure 4.2B:

Selected electropherograms of individuals from the 4CRD family. The left electropherogram is for the heterozygous carrier (Individuals III:1) and the right electropherogram is of the patient IV:1 that is homozygous for the G-T transversion in exon 13 of the RPRGRIP1 gene.

The same mutation was also found in 5CRD and 10CRD families.
4.4 DISCUSSION:

Homozygous mutations in the *RPGRIP1* gene have been reported in a panel of unrelated patients with Leber congenital amaurosis (LCA; Dryja *et al.*, 2001). In most of these cases the mutations result in a premature termination codon. To date *RPGRIP1* is the only gene that has not been associated with any other retinal disease phenotypes except LCA. LCA represents the severe end of a spectrum of inherited retinal dystrophies while cone rod dystrophy is a milder condition. It has been suggested that mutations that cause residual *RPGRIP1* activity may lead to phenotypes such as RP or CRD that are less severe compared to LCA (Cremers *et al.*, 2002). Mapping of two Pakistani families with cone rod dystrophy to the *RPGRIP1* locus supports this hypothesis. The identification of two novel disease associated mutations also indicates allelic heterogeneity of the *RPGRIP1* gene. Both novel mutations are in exons encoding domains of *RPGRIP1* that are reported to be involved in interaction with the *RPGR* gene product (Dryja *et al.*, 2001).

Prosite (http://us.expasy.org/tools/scanprosite/) scan predicted the occurrence of an additional, more efficient, casein kinase II phosphorylation site in the Ala547Ser mutated protein, the significance of which is unknown. The second mutation (Arg827Leu) was found in the major calcium-dependent membrane-binding module, the CK2 domain of the *RPGRIP1* protein. However, the prediction did not indicate any change in the 3D structure of the domain. Functional analysis of this protein would be required to demonstrate the role of these mutations in retinal dystrophies.
4.5 REFERENCES:


CHAPTER 5

NOVEL ASSOCIATION OF RP1 GENE MUTATIONS WITH AUTOSOMAL RECESSIVE RETINITIS PIGMENTOSA.

5.1 INTRODUCTION:

Retinitis pigmentosa (RP) is the most prevalent hereditary retinal degenerative disease. To date approximately 150 loci and mutations in more than 100 genes have been identified as the cause of various types of RP ([http://www.sph.uth.tmc.edu/RetNet/](http://www.sph.uth.tmc.edu/RetNet/)). The gene for human oxygen-regulated photoreceptor protein (RP1) encodes a protein of 2156 amino acids that is localized in the connecting cilia of both rod and cone photoreceptors (Liu et al, 2002). The RP1 protein is required for the morphogenesis of the outer segments of the photoreceptor cells (Gao et al, 2002; Liu et al, 2003). Several laboratories have found mutations in the RP1 gene as the cause of autosomal dominant retinitis pigmentosa (adRP: Bowne et al, 1999; Pierce et al, 1999; Payne et al, 2000). However, association of the RP1 gene with recessive RP has never been reported.

The aim of this study was to map the disease locus for three consanguineous Pakistani families suffering from arRP. A genome-wide search mapped the disease locus to chromosome 8q11 that is known to harbour the RP1 gene. The results show, for the first time, that in these families a form of arRP is caused by homozygous mutations of the RP1 gene. These mutations were found in all the parents and some of the siblings who had normal vision (carriers) in a heterozygous state, they were not found in any of a panel of 100 controls.
5.2 PATIENTS AND METHODS:

Three consanguineous Pakistani families (442RP, 452RP and 336RP) suffering from autosomal recessive RP were studied (Fig. 5.1A, 5.2 and 5.3A). The patients had night blindness since early childhood and progressive deterioration of vision with age. All the patients were completely blind by the age of 12-15 years in the case of the 442RP and 452RP families and 17-18 years in the 336RP family. Fundoscopic examination and electroretinographic (ERG) analyses were carried out on all the patients, their parents as well as unaffected normal siblings.

Fundoscopic examination of the affected individuals, V:3 (22y), V:5 (15y) and V:12 (18y) from the 442RP family; individuals IV:1 (25y), IV:6 (12y) and IV:9 (18y) from the 452RP family was carried out. All the patients had attenuation of blood vessels, pale optic disc, stippling of macula with no bony spicules. Parents from all branches of these families and some of their normal siblings were also examined. None of the (normal) individuals had any sign of RP. ERG examination showed nonrecordable or reduced electroretinographic response in all the patients. Their unaffected parents (ages ranged from 35-60 years) had normal ERGs (rod and cone function). Clinical examination of patients from the 336RP family also revealed typical features of RP, including pigment deposition, attenuation of retinal blood vessels and pale optic disc. Patients IV:2 and IV:4 (aged 17y & 22y respectively) had pigment deposition in the equatorial as well as macular region at the time of investigation. Fundoscopic examination of the unaffected father (40y) showed neither pigment deposition nor abnormality of the retina.

For genetic analysis, peripheral blood samples were collected from all members of the families with informed consent. Control samples were also collected
from 100 ethnically matched, unrelated, normal Pakistani individuals. Patients were examined at the retina clinic of one of the following hospitals: (1) KRL General Hospital, Islamabad; (2) Al-Shifa Trust Eye Hospital, Rawalpindi; and (3) Christian Hospital, Taxila.

5.2.1 Linkage analysis:

Genomic DNA was extracted from whole blood using the standard phenol-chloroform extraction procedure. DNA was amplified using specific primers for polymorphic microsatellite markers (Research Genetics, Version 8) for known genes and loci associated with various types of retinal degeneration according to the conditions described in the chapter 2.

5.2.2 Mutation Detection:

Exon specific primers were designed from the genomic sequence of the RP1 gene (Table 5.1). PCR was performed in a 50μl reaction volume using 1μg of genomic DNA. Heteroduplex analysis was performed using an automated denaturing high performance liquid chromatography (DHPLC) instrument (WAVE DNA fragment analysis system, Transgenomic, Crewe, UK: Underhill et al, 2000). Samples were prepared by denaturing and reannealing of the unpurified PCR products. The temperature conditions required for the successful resolution of heteroduplexes were obtained from the website (http://insertion.stanford.edu/melt.html).

Following heteroduplex analysis, all family members were sequenced in the forward and reverse directions using a commercially available kit (Big Dye, ABI) and the products were analyzed on an ABI Prism 377 automated DNA sequencer. To examine the possibility that the mutations are polymorphisms, a
panel of 100 normal individuals was also analyzed for RP1 gene mutations, initially by DHPLC followed by direct DNA sequencing.
TABLE 5.1:
PRIMER PAIRS AND PCR CONDITIONS FOR MUTATION SCREENING OF THE RP1 GENE:

<table>
<thead>
<tr>
<th>EXON #</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>PCR product size (bp)</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GTCTGGTGATTAGCATCACCATGG</td>
<td>GTAACAGTTAAGACAGAACAACACAG</td>
<td>309</td>
<td>55°C X 1.5mM Mg⁺²</td>
</tr>
<tr>
<td>2.1</td>
<td>CTGTGAATATCCCTGGATGTCTG</td>
<td>GGCTGCACCTTCTTCCTGCCGTCGGG</td>
<td>457</td>
<td>58°C X 1.5mM Mg⁺²</td>
</tr>
<tr>
<td>2.2</td>
<td>GAGCTGGAGGACGGCGAGTCC</td>
<td>GGCCACCATTATCATTCCACCACAC</td>
<td>410</td>
<td>68°C X 1.5Mm Mg⁺²</td>
</tr>
<tr>
<td>3</td>
<td>GTCTTTCAGCCTAGGAGGTGG</td>
<td>GAAGCATGGAATTGCGCGTCGC</td>
<td>358</td>
<td>58°C X 1.5mM Mg⁺²</td>
</tr>
<tr>
<td>4.1</td>
<td>CATAGAGCTCAGAAACATATCAGT</td>
<td>TCCTCTTTATTTCTGAATCG</td>
<td>438</td>
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</tr>
<tr>
<td>4.2</td>
<td>CAAGACGGCCTATGACAGGTGGA</td>
<td>GTCTCAGCTCTTCTTAGTGCCAG</td>
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<td>60°C X 1.5mM Mg⁺²</td>
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<tr>
<td>4.3</td>
<td>CCAGGGAACTCAAGACCA</td>
<td>TCCTCAAACATCTCTTGACCCT</td>
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</tr>
<tr>
<td>4.4</td>
<td>CAAGTGCAATAAGTGCTGGT</td>
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</tr>
<tr>
<td>4.5</td>
<td>CTCGACAGCAAGCATAAAATTCC</td>
<td>GCTCAAGGATGTAAATACATG</td>
<td>455</td>
<td>58°C X 1.5Mm Mg⁺²</td>
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<tr>
<td>4.6</td>
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<td>TGGGATCCATATCCTAAAC</td>
<td>465</td>
<td>56°C X 1.5Mm Mg⁺²</td>
</tr>
<tr>
<td>4.7</td>
<td>CAGCTCGAGGTATGAGAAATG</td>
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<td>411</td>
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</tr>
<tr>
<td>4.8</td>
<td>GCAGACAAATCGCGACAGAG</td>
<td>ACAGCAGTTTTCAAGTC</td>
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<tr>
<td>4.9</td>
<td>GCACATAGCTATCACAGAGG</td>
<td>GCTCCCTCATAGGTATGGTTC</td>
<td>474</td>
<td>60°C X 1.5Mm Mg⁺²</td>
</tr>
<tr>
<td>4.10</td>
<td>GGAGAGGTCTGTCTCTCCCTG</td>
<td>CACTGATGTGATGCTGTGTCG</td>
<td>478</td>
<td>60°C X 1.5Mm Mg⁺²</td>
</tr>
<tr>
<td>4.11</td>
<td>GATATGGGAAGAACCACCGGACT</td>
<td>ACCATCAGAAGTCACTGGAC</td>
<td>463</td>
<td>60°C X 1.5Mm Mg⁺²</td>
</tr>
<tr>
<td>4.12</td>
<td>GTATTTCAGTCTCCCTCTG</td>
<td>GTACAGGATCTTCAATAATGCAC</td>
<td>457</td>
<td>56°C X 1.5Mm Mg⁺²</td>
</tr>
<tr>
<td>4.13</td>
<td>GATGTAGTTGCTGGAGGAGG</td>
<td>CTGACTGATGACTACCTATCTC</td>
<td>469</td>
<td>60°C X 1.5Mm Mg⁺²</td>
</tr>
<tr>
<td>4.14</td>
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<td>4.15</td>
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</tr>
<tr>
<td>4.16</td>
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<td>473</td>
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<tr>
<td>4.17</td>
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<td>CTATGAGATTGTGCCTGGATATC</td>
<td>290</td>
<td>56°C X 1.5Mm Mg⁺²</td>
</tr>
</tbody>
</table>
5.3 RESULTS:

Exclusion studies of known RP loci were carried out for the three-arRP families. Homozygosity was observed for markers D8S285 and D8S1815 for all the patients (Figure 5.1A, 5.2 and 5.3A). This locus contains the RP1 gene that has previously been reported to be associated with dominant RP.

Since most of the mutations identified previously are clustered in a small region, the 5' region of exon 4 was screened (Dietrich et al, 2002). DHPLC analysis showed heteroduplex peaks for the unaffected parents and some of the siblings of the patients in both the 442RP and 452RP families. Samples of all affected individuals showed homoduplexes. All the family members were then sequenced to identify the change in exon 4. Sequencing analysis revealed that patients had a homozygous C→T substitution at nucleotide 1118 (Fig. 5.1B). Parents of the affected individuals were heterozygous for this change. This substitution causes a missense codon 373 that codes for isoleucine (ATA) instead of threonine (ACA) in the mutated protein product. The normal family members including their parents (carriers) who are heterozygous for the Thr373Ile change have normal vision with no signs of RP. Eukaryotic Linear Motif (ELM; http://elm.eu.org/) scans showed that the T373I missense mutation abolishes the glycogen synthase phosphorylation recognition site (GSK3) resulting in a conformational change in the mutated protein. To check if there is another mutation in this gene associated with the disease phenotype in both these families, the remaining three coding exons of the RP1 gene were also sequenced. No other disease-associated change was found in any patient of the 442RP and 452RP families. None of the 100 normal controls carried the T373I change in the homozygous state, although some heterozygous individuals were observed. Payne et al (2000)
have reported this change to cause adRP. However, Berson et al (2001) categorized this mutation as nonpathogenic because it did not cosegregate with the disease phenotype in one of their adRP families. They suggested that mutations in RP1 might be recessive, causing RP or some other retinal disease. Our data shows that the homozygous T373I change causes arRP in consanguineous families.
Figure 5.1A:

Pedigree of 442RP family. Normal and mutated alleles of each individual are labeled (+) and (M) respectively.
Figure 5.1B.

Selected electropherogram showing a homozygous C-T substitution at nucleotide 1118 (Thr373Ile) in patients from families 442RP and 452RP.
Figure 5.2:
Pedigree of 452RP family. Normal and mutated alleles of each individual are labeled (+) and (M) respectively.
In the unaffected members of the third family (336RP), heteroduplex peaks were also observed for another fragment of exon 4. Sequencing analysis of all family members showed a homozygous 4bp insertion (1461-1465insTGAA) in the patients (Fig. 5.3B). This insertion adds a stop codon immediately after nucleotide 1461 resulting in a severely truncated protein of 487 amino acids instead of 2156 amino acids. This 4bp insertion was present in a heterozygous state in some unaffected members of the family including the parents of the patients. Subsequently, the panel of normal controls was screened for this insertion. None of the 100 normal individuals carried this change.
Figure 5.3A:

Pedigree of 336RP family. Normal and mutated alleles of each individual are labeled (+) and (M) respectively.
Figure 5.3B.

Selected electropherogram showing a homozygous 4bp insertion introducing a stop codon at nucleotide 1461 in patients from the 336RP family
A random panel of 150 RP patients was also screened for \textit{RP1} gene mutations. A heterozygous single base pair G→A substitution at nucleotide position 2005 was found in one patient (Fig. 5.4). This substitution replaces alanine (GCC) with threonine (ACC) at codon 669. This change was not found in any of the 100 normal individuals. In addition to these disease-causing mutations, several sequence variants and polymorphism were also found in this study (Table 5.2). These results show that two polymorphisms, N985Y and P1205P are prevalent in the Pakistani samples.
Figure 5.4.

Selected electropherogram showing a heterozygous G-A mutation at nucleotide 2005 (Ala669Thr) in a single individual from the RP panel.
Table 5.2:
Polymorphisms found in the RP1 gene in families and random unrelated Pakistani controls.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Base Change</th>
<th>Amino Acid Change</th>
<th>Number of Patients/Families</th>
<th>Number of Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>4(6)</td>
<td>2615G→A</td>
<td>R872H</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>4(6)</td>
<td>2644A→G</td>
<td>I882V</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>4(7)</td>
<td>2953A→T</td>
<td>N985Y</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>4(9)</td>
<td>3615A→T</td>
<td>P1205P</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>4(12)</td>
<td>5008G→A</td>
<td>A1670T</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>4(12)</td>
<td>5071T→C</td>
<td>S1691P</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>4(13)</td>
<td>5175A→G</td>
<td>Q1725Q</td>
<td>2</td>
<td>-</td>
</tr>
</tbody>
</table>
5.4 DISCUSSION:

Previous studies have identified 21 different disease-causing mutations that are responsible for 6-10% cases of adRP in ethnically diverse populations (Bowne et al, 1999; Payne et al, 2000; Berson et al, 2001). None of these mutations were found in the Pakistani samples. Identification of only one mutation in the unrelated 150 RP patients and two mutations with recessive RP among a large number of patients and families shows that $RP_1$ gene mutations are not a major cause of RP in Pakistan.

Dominant RP caused by mutations in the $RP_1$ gene often shows late onset of the disease phenotype, usually by the third decade of life. Pierce et al (1999) observed that patients suffering from adRP who were heterozygous for the RP1 mutation had classic, less severe adRP phenotype with late onset of disease. By contrast patients that were homozygous for the mutation in the same family had substantially more severe form of the disease and the age of onset was variable (Sullivan et al, 1999). All three recessive Pakistani families presented here showed the severe form of RP with early onset.

It is well known that Mendelian disorders caused by the dysfunction of a single gene have a wide heterogeneity of disease phenotypes (Bell, 2004). RP is a heterogeneous group of retinal disorders and mutations within a single gene are known to cause different clinical phenotypes (Rosenfeld et al, 1992; Dryja et al, 1990; Gerber et al, 2001; Hameed et al, 2002). The work presented here shows, for the first time, that arRP is also caused by (homozygous) mutations in the $RP_1$ gene. This could be due to centuries of consanguineous marriages in this gene pool, altering the penetrance of the mutated gene in the heterozygous state.
5.4 REFERENCES:


RetNet. University of Texas-Houston Health Science Center; available in the public domain at http://www.sph.uth.tmc.edu/Retnet/disease.htm


CHAPTER 6

IDENTIFICATION OF NOVEL MUTATIONS IN SEMA4A GENE ASSOCIATED WITH RETINAL DEGENERATIVE DISEASES.

6.1 INTRODUCTION:

During the development of the nervous system of multicellular organisms, several proteins play a role in communication between the cells and their local environment. Among those known to be important for axon guidance are ephrins, netrins, slits and semaphorins (Bagri and Tessier-Lavigne, 2002).

Semaphorins are a large family of trans-membrane proteins. The whole family shares a conserved domain (Sema) at the NH$_2$-terminal. The semaphorin family is further divided into seven subclasses based on their functional domains and sequence similarity. Classes 1 and 2 are found in invertebrates whereas classes 3 to 7 are found in vertebrates (Tessier-Lavigne and Goodman, 1996; Dickson, 2002). Semaphorin proteins are involved in a variety of biological mechanisms, such as organogenesis, angiogenesis, and neuronal development (Kawasaki et al, 1999; Feiner et al, 2001; Fiore and Puschel, 2003; Kumanogoh and Kikutani, 2003). It has been reported that semaphorin subclasses, Sema4A and D possess similar structures and are involved in cell-cell communication between T cells and antigen-presenting cells in the immune response (Shi et al, 2000; Kumanogoh et al, 2002). Sema4a provides a co-stimulatory signal for T cell priming and regulation (Kumanogoh et al, 2005).

The gene for SEMA4A encodes a transmembrane protein comprising 760 amino acids. SEMA4A contains a signal peptide preceding a conserved
semaphorin domain (aa 64-478), followed by a PSI domain (aa 496-580), an Ig-like domain (aa 570-630), a transmembrane domain (aa 680-702), and a short (AA 703-760) cytoplasmic tail. SEMA4A is highly expressed in the brain and eye and during embryonic development, it is expressed in the ganglion cells, inner retinal neurons and retinal pigment epithelial (RPE) cells, particularly at the time of contact between photoreceptors and RPE during development (Rice et al, 2004).

Recently in a study on a mouse model, it was shown that disruption of the Sema4A gene results in severe retinal degeneration, attenuation of retinal blood vessels and depigmentation of the retinal pigment epithelium. The disruption also affects the physiological function of both rod and cone photoreceptor cells. Hence, it had been suggested that Sema4A functions as a transmembrane ligand for a receptor present on photoreceptor cells (Rice et al, 2004).

The gene for the human SEMA4A protein is present on chromosome 1q22 (http://www.ensembl.org). To date no study has been published showing an association between SEMA4A gene mutations and human retinal degeneration. To investigate its association with human retinal degeneration, mutation screening of SEMA4A gene was carried out on 190 unrelated patients suffering from a variety of eye diseases. Here we report the first observation of the involvement of the SEMA4A gene mutations causing retinitis pigmentosa (RP) and cone rod dystrophy (CRD).

6.2 SUBJECTS AND METHODS:

Blood samples were obtained with the informed consent of all subjects. Leukocyte DNA was extracted from peripheral blood of 190 unrelated patients
suffering from various retinal diseases, including RP, CRD and LCA. Blood samples were also collected from 100 ethnically matched control subjects.

All patients studied were of Pakistani origin belonging to various northern ethnic groups such as Pathans, Punjabis and Kashmiris. Diagnosis was made on the basis of previous history of patients and clinical notes from their childhood examinations and fundoscopy, done at the time of sample collection.

RP patients initially had night blindness followed by complete blindness. Fundoscopic examination revealed the clinical features of retinitis pigmentosa that includes typical bony corpuscles-type pigmentation, deposited mainly in the equatorial and peripheral region. Attenuated blood vessels were also seen towards the periphery. The macula was clear in those patients who were at early stages of the disease. Patients were labelled CRD if they had progressive loss of visual acuity and colour vision followed by night blindness and loss of peripheral vision. Most of the CRD patients had severe photophobia and epiphora in bright light. Fundoscopic examination revealed high degree of fundus granularity with marked macular degeneration and significant level of peripheral retinal pigmentation. Patients presented blind by birth or during infancy were considered to have LCA.

To identify disease-associated mutations in the SEMA4A gene, DNA samples of patients were screened by SSCP, followed by direct DNA sequencing. From the SEMA4A gene sequence (accession number, NM_022367) exon-specific intronic primers were designed covering the splice sites on both ends of the exons (Table 6.1). The SEMA4A gene consists of 15
exons. Each exon was individually amplified from genomic DNA samples by PCR in a 50μl reaction volume under standard PCR conditions (Table 6.1).

For SSCP, PCR products were electrophoresed on a 12% non-denaturing resolving gels in Tris-glycine buffer at 70-80V, overnight. The 12% resolving gel was prepared by adding 12ml of a 30% polyacrylamide stock solution (30:1, acrylamide: bisacrylamide), 6ml of 5xTGB (125mM Tris pH8.0, 0.96M glycine) and the volume adjusted to 30ml with deionized water. The bands were visualized by silver staining (Bassam et al, 1991).

Samples that showed a mobility shift in SSCP analysis were sequenced. Genomic DNA fragments containing the coding sequence and the flanking splice-junction consensus sequences of each exon were amplified by PCR. The amplified fragments were purified on QIAquick® spin columns (QIAGEN) and subjected to sequence analysis in both forward and reverse directions. For each sample the sequencing reaction was set up using the Big Dye Terminator cycle sequencing Kit (ABI). The products were separated by electrophoresis and analysed using an ABI 377 automated DNA sequencer. A missense, in-frame change or compound heterozygous mutation was considered pathogenic if found only in the patients and not in any of the 100 normal controls.
<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer sequence (5’→ 3’)</th>
<th>PCR product size (bp)</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1F</td>
<td>CCACCAACTTCCGGCCCAAGCC</td>
<td>372</td>
<td>1.5mM MgCl₂ @60°C</td>
</tr>
<tr>
<td>1R</td>
<td>CCGGCTCCCAGCCTCTCGAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2F</td>
<td>CCTGCCACCAATACACACAGC</td>
<td>389</td>
<td>1.5mM MgCl₂ @60°C</td>
</tr>
<tr>
<td>2R</td>
<td>TGTCCTGTGGTCTCCATATCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3F</td>
<td>TGCATCTAGCTGCAAGGGCC</td>
<td>375</td>
<td>1.5mM MgCl₂ @67°C</td>
</tr>
<tr>
<td>3R</td>
<td>TTATTCATGCAAGGCAGCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4F</td>
<td>ATCAGCATGTCACTAACCACC</td>
<td>320</td>
<td>1.5mM MgCl₂ @57°C</td>
</tr>
<tr>
<td>4R</td>
<td>AACATAATCCAGGAGATAACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5F</td>
<td>AGCATTACCTAGCCTTCTCC</td>
<td>299</td>
<td>1.5mM MgCl₂ @58°C</td>
</tr>
<tr>
<td>5R</td>
<td>CATCTGGAAGGCCAGAGTTTCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6F</td>
<td>AGACCAATTTTCCCTCTATGCCC</td>
<td>368</td>
<td>1.5mM MgCl₂ @59°C</td>
</tr>
<tr>
<td>6R</td>
<td>AGCATCCTTCAACTTCAGTTTCC</td>
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<td></td>
</tr>
<tr>
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<td>1.5mM MgCl₂ @60°C</td>
</tr>
<tr>
<td>7R</td>
<td>GAAAGTTCATCAAGGAAACGAGG</td>
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<td></td>
</tr>
<tr>
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<td>GAGGAAGCCTGTGTGCCTTGG</td>
<td>283</td>
<td>1.5mM MgCl₂ @62°C</td>
</tr>
<tr>
<td>8R</td>
<td>TATTTTCTCCTCCAGTTAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9F</td>
<td>GGGTGCTAGAGTCGTCAAGG</td>
<td>623</td>
<td>1.5mM MgCl₂ @60°C</td>
</tr>
<tr>
<td>9R</td>
<td>GAAAGTGTGACTGAGGTGCTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon</td>
<td>Primer sequence (5'→ 3')</td>
<td>PCR product size (bp)</td>
<td>PCR conditions</td>
</tr>
<tr>
<td>------</td>
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<td>CTCCCTTGGGCCCTTATCAACAC</td>
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<tr>
<td>10R</td>
<td>GGCATAGTAATGCCCAATAAA</td>
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<tr>
<td>11F</td>
<td>CCACCCTGAAATGAGGACTGCC</td>
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<td>11R</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>12F</td>
<td>CCGCGGCCAGTACATACACC</td>
<td>335</td>
<td>1.5mM MgCl₂ @68°C</td>
</tr>
<tr>
<td>12R</td>
<td>CCCAGCAGCTGGTGAGGCGC</td>
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<td></td>
</tr>
<tr>
<td>13F</td>
<td>CCTGACCATCAATGGGCTTTC</td>
<td>441</td>
<td>1.5mM MgCl₂ @60°C</td>
</tr>
<tr>
<td>13R</td>
<td>CCATCCCTGGAGGAAAACCTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14F</td>
<td>TTCCGCCTCTCCTCTCTGTTC</td>
<td>361</td>
<td>1.5mM MgCl₂ @55°C</td>
</tr>
<tr>
<td>14R</td>
<td>TCGGGGTGCAGATCTCAAAGCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15.1F</td>
<td>GGGCTGGGTCCAAGATAGGC</td>
<td>437</td>
<td>1.5mM MgCl₂ @70°C</td>
</tr>
<tr>
<td>15.1R</td>
<td>GACTGCTGGCCAGCCAGGGCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15.2F</td>
<td>CTTTCATTACCCCTGTGATCTCC</td>
<td>531</td>
<td>2.5mM MgCl₂ @65°C</td>
</tr>
<tr>
<td>15.2R</td>
<td>CAGAGTAGCAGAAGCTCCTCCT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F = Forward, R = Reverse
6.3 RESULTS:

Our panel of patients include 135 patients with RP, 25 patients with CRD and 30 patients suffering from LCA. For mutation detection SSCP analysis was carried out with the whole panel and direct DNA sequencing was done for the selected individuals from SSCP analysis data. The results are summarised in table 6.2.
Table 6.2

Summary of *SEMA4A* gene sequencing results.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Base Change</th>
<th>Codon</th>
<th>Amino Acid</th>
<th># patients/controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathogenic changes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1033G→C</td>
<td>GAC-CAC</td>
<td>D345H</td>
<td>4 patients</td>
</tr>
<tr>
<td></td>
<td>1049T→G</td>
<td>TTT-TGT</td>
<td>F350C</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>2138G→A</td>
<td>CGG-CAG</td>
<td>R713Q</td>
<td>4 patients</td>
</tr>
<tr>
<td>Nonpathogenic changes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>39C→A</td>
<td>CTC-CTA</td>
<td>L13L</td>
<td>6 patients</td>
</tr>
<tr>
<td>8</td>
<td>762T→C</td>
<td>TTT-TTC</td>
<td>F254F</td>
<td>11 patients</td>
</tr>
<tr>
<td>Intron 10</td>
<td>CAdel, 26bp downstream of exon 10</td>
<td></td>
<td></td>
<td>13 patients &amp; 11 controls</td>
</tr>
<tr>
<td>15</td>
<td>1716C→T</td>
<td>CCC-CCT</td>
<td>P572P</td>
<td>9 patients</td>
</tr>
</tbody>
</table>
During SSCP analysis for exon 10, two types of variant bands were seen in 20 different samples. These selected samples were subsequently sequenced to find out the exact sequence change that corresponds to the mobility shift in the SSCP gels. Sequencing analysis revealed two heterozygous changes in 4 out of 20 patients. The first change was a heterozygous G to C substitution at nucleotide 1033. This change replaces aspartic acid (GAC) with histidine (CAC) at codon 345. This change results in a p.D345H mutation found to be conservative in nature. The second change was a T to G substitution at nucleotide 1049. This change in codon 350 changes the amino acid from phenylalanine (TTT) to cysteine (TGT) results in a non-conservative p.F350C mutation (Fig 6.1). Both p.D345H & p.F350C mutations were identified in four patients (RODS002, 006,067,119). Of these, two were diagnosed to have RP and other two were suffering from CRD. It is noteworthy that all the patients had both mutations in heterozygous condition and none had only one. To confirm the pathogenic nature of these mutations other family members of one of the CRD patient (RODS006) were subsequently sequenced. The results revealed that he inherited the p.D345H mutation from his father while the p.F350C mutation came from his mother (Fig 6.3A). Upon clinical examination both the parents appeared to be normal. Clinical examination of this patient revealed typical phenotype of cone-rod dystrophy. He had a progressive loss of visual acuity and colour vision followed by night blindness and loss of peripheral vision. Fundoscopy showed high degree of fundus granularity with marked macular degeneration and peripheral retinal pigmentation. None of the normal controls had either one of
these mutations. It can therefore, be inferred that compound heterozygous mutations cause this disease phenotype.

The remaining 16 patients (out of the 20 individuals that showed a mobility shift in SSCP analysis) had a 2 base pair deletion in intron 10, 26bp down stream of exon 10. In addition a large number of samples from the normal controls were also identified as having the above-mentioned 2 base pair deletion. This polymorphic deletion was heterozygous in all the samples that were examined. This deletion was considered nonpathogenic because it was found in both the patients and the control subjects. In addition three isocoding substitutions (C→A, T→C and C→T) were also identified in exon 2, 8 and 15 respectively.

Another mutation was found in exon 15 in three patients suffering from RP and one patient with congenital blindness (Fig 6.3B). This heterozygous G to A transition mutation at nucleotide position 2138 changes the codon 713 for arginine (CGG) with glutamine (CAG). Sequence analysis of the family members of an RP patient (RODS52) confirmed that R713Q mutation was segregating with the disease phenotype (Fig 6.2) with an autosomal dominant mode of inheritance. This mutation was not present in the 100 ethnically matched control subjects.
Figure 6.1:

Selected electropherogram (forward and reverse) of the patient showing compound heterozygous G→C and T→G substitutions in exon 10.
Figure 6.2:

Selected electropherogram (forward and reverse) of the patient showing heterozygous G→A substitutions in exon 15.
Figure 6.3A

G/C  
T/T  

G/G  
T/G  

→ RODS006

C/G  
T/G  

G/G  
T/T  

Figure 6.3B

G/G  

G/A

→ RODSS54

G/A  
G/G  
G/A  
G/A

Figure 6.3

Pedigrees of families of (A) a CRD patient with compound heterozygosity for the D345H, & F350C (G→C and T→G) mutations and (B) of an autosomal dominant RP patient in which sequencing analysis showed the segregation of the R713Q (G→A) mutation. Arrows indicate index cases.
6.4 DISCUSSION:

Of the 190 patients analysed, three novel point mutations were found in the *SEMA4A* gene. These mutations could be considered pathogenic for two reasons. First, these mutations were not observed in any of the normal or the 100 control subjects. Second, the p.D345H & p.F350C mutations identified in this human study occur in the conserved semaphorin domain. In the mouse model, it has been shown that disruption in this domain causes severe retinal degeneration including attenuated retinal blood vessels and depigmentation (Rice *et al.*, 2004). However, the R713Q mutation, found in the RP and congenitally blind patients, occurs in the cytoplasmic tail. This mutation probably disrupts the signal that activates the biochemical pathways required for the normal function of the cell.

Multiple sequence alignments using Clustal analysis showed that R713Q mutation is a conserved substitution and D345H mutation is a semi conserved substitution in which an acidic amino acid is changed into a basic amino acid (http://www.ebi.ac.uk/clustalw/index.html).

The novel identification of these mutations in patients as a cause of various retinal degenerations could be helpful to further understand the function of *SEMA4A* in the visual system and the role that it plays in the signalling mechanism to control the development of the outer retina.
6.6 REFERENCES:


CHAPTER 7
GENERAL DISCUSSION:

Of the 20,000-25,000 genes that altogether make a human, mutations in more than 1,900 genes have been identified as the cause of hereditary disorders (OMIM, statistics for Aug 2006). More than 1,500 known Mendelian diseases have been reported in the literature, for which the genetic defect remain unidentified.

The work presented in this thesis examines ocular diseases that are known to constitute a large portion of the known Mendelian disorders. Over the last few decades, advancements in the field of molecular biology have contributed to a better understanding of the genetics of ocular disorders (Bok, 2007). Although we are far from a genetic cure to these diseases, there is a great potential in the therapeutic interventions that are constantly under investigation.

This study was carried out to find genetic causes of retinal degenerative diseases that are prevalent in the Pakistani population. Being a Muslim country, consanguineous marriages are a part of our culture and tradition. It is estimated that approximately 20%-50% marriages are consanguineous involving about one billion people worldwide (Bittles, 2001). As a consequence, large inbred families suffering from a variety of diseases can be found very commonly in Pakistan.

Inherited retinal dystrophies constitute a large portion of the blind population of Pakistan. Multigenerational families suffering from inherited retinal dystrophies are found in almost all areas of Pakistan. These families are best suited for homozygosity mapping of disease causing genes by linkage analysis.
For this study a nation-wide search was carried out to ascertain affected families suffering from retinitis pigmentosa, cone rod dystrophy and Leber's congenital amaurosis. Blood samples were collected with informed consent. In addition a large cohort of patients suffering from these three disorders were also collected to make a panel of retinal dystrophy samples. In most of the families the mode of inheritance was autosomal recessive. A few small families with dominant inheritance were also identified and included in the retinal dystrophy panel.

Detailed clinical analyses, including fundoscopy and ERG, were carried out on each individual either at the KRL Hospital, Islamabad or the Al-Shifa Trust Eye Hospital, Rawalpindi.

For molecular genetic studies, each family was analyzed for the known retinal dystrophy loci. Upon indications of linkage to any known locus, mutation screening/sequencing was carried out to find the disease causing mutation. In cases where all known loci were excluded, a genome wide search was carried out. For this microsatellite markers spanning all the chromosomes at intervals of approximately 10cM were used. Upon indication of linkage on a specific chromosome, other polymorphic markers within the region were screened in order to define the critical disease interval. Two-point and/or multi-point LOD scores were calculated to ascertain linkage and the region was subsequently screened for candidate genes using databases such as Ensembl, NCBI and GenBank. Single strand conformational polymorphism (SSCP) and denaturing high performance liquid chromatography (DHPLC) analyses of the putative disease causing defective region were also carried out for mutation detection in
the retinal dystrophy panel. Direct sequencing was then performed on variant bands to find out the disease causing mutation. A summary of the results obtained is given below.

Two families suffering from autosomal recessive retinitis pigmentosa (arRP) with para-arteriolar preservation of the retinal pigment epithelium (PPRPE) and one family with leber congenital amaurosis (LCA) were found to have linkage at chromosome 1q (Z max 3.79, θ = 0) with marker D1S413. This region of chromosome 1 (1q31-32.1) has been reported to have the CRB1 gene and is known to be associated with the RP12 type of retinitis pigmentosa (den Hollander et al., 2001; Lotery et al., 2001a; Lotery et al., 2001b). Heteroduplex analysis of all 11 exons of the CRB1 gene was carried out using DHPLC with samples from patients and unaffected carriers of all the three families. Heteroduplex mismatches were obtained in the samples of carriers. Sequencing analysis of the CRB1 gene revealed three novel disease-causing mutations in each family. A homozygous G to A substitution at codon 846 in exon 7 of the CRB1 gene was found in the 3330RP family. This mutation replaces a glycine with an arginine. Another T to C substitution at codon 1116 in exon 9 changed leucine with a proline in the second 111RP family. The 010LCA family had a homozygous T to C substitution at codon 989 that changed iso-leucine with a threonine residue. All these mutations segregated with the disease phenotype in the families and none was found in the ethnically matched normal control population. All the identified CRB1 mutations are in the laminin G domain of the crb1 protein, which indicates the functional importance of this domain.
The *CRB1* gene is the human homolog of the Drosophila crumbs gene. In Drosophila, crumbs is required for the formation of adherens junctions (AJ) between the cells (Grawe *et al.*, 1996; Tepass, 1996). The adherens junctions are present between the photoreceptor cells and Muller glial cells and are packed together in the retinal outer nuclear layer (ONL). They are crucial in the establishment and maintenance of apical-basal polarity and cell adhesion of the photoreceptor cells. Crumbs is localized in the sub-apical region (SAR) of the photoreceptor and is an essential component of the intracellular scaffold for the assembly of the protein complex at the AJ (Pelliika *et al.*, 2002). Though the exact role of *CRB1* in retinal dystrophies is still unclear, a mouse model of Crb1, retinal degeneration 8 (rd8) was observed to developed irregularities at the outer limiting membrane and the loss of photoreceptor cells (Mehalow *et al.*, 2003). Another study using Crb1<sup>−/−</sup> mouse revealed a crucial role of the Crb1 in the scaffolding complex thus maintaining a single organized layer of photoreceptor during light exposure. In the absence of Crb1, the adhesion between the photoreceptors and the Muller glial cells was temporarily lost resulting in drastic structural and functional changes (Van de Pavert *et al.*, 2004).

Another LCA family (011LCA) was found to be linked to chromosome 17. Significant LOD scores were obtained with markers D17S1294 and D17S796 at 17p13.1. Two genes, *GUCY2D* and *AIPL1* were reported to reside in this region. Using DHPLC analysis, heteroduplex mismatches were found in exon 2 of the *AIPL1* gene in the carriers of the family. Sequencing analysis of exon 2 of *AIPL1* gene revealed a C to A transversion in a homozygous state in all the affected family members. This mutation at codon 116 replaces the amino acid threonine.
with asparagine. Sequencing of other exons of the AIPL1 gene as well as GUCY2D gene revealed no other disease associated mutation. In the past, many disease causing mutations were identified in Pakistani LCA families indicating a high prevalence of AIPL1 gene mutations in Pakistan (Sohocki et al, 2000; Damji et al, 2001). Mutations in the AIPL1 gene are reported to cause 10% of all the LCA cases worldwide (Sohocki et al, 2000; Sohocki et al, 2001; Weleber, 2002). The Aipl1 protein is expressed in the developing cone and rod photoreceptor precursor cells. However, in the adult retina the expression of AIPL1 protein was restricted only to the rod photoreceptor cells suggesting its role in the viability and maintenance of rod cell (van der Spuy et al, 2002, 2003, 2005). The Aipl1 protein contains three tetratricopeptide (TPR) repeat motifs, that are 34 amino acids in length. They are thought to act as interfaces for protein-protein interactions and are commonly found in proteins having a protein chaperone activity (Das et al, 1998).

Two multigenerational families (1CRD and 4CRD) suffering from cone-rod dystrophy were analysed. None of these families linked to the known CRD loci, indicating the involvement of a novel gene. During the screening of known loci for other retinal degenerations, significant linkage was obtained with marker D14S1023 (Zmax = 5.17 and 4.21 for 1CRD and 4CRD respectively). This locus of chromosome 14q11 harbours the RPRGRIPI gene that is known to cause LCA (Dryja et al, 2001; Gerber et al, 2001). DHPLC analysis followed by sequencing of the coding exons of the RPRGRIPI gene were performed for both the families. The results revealed a homozygous G to T point mutation in exon 16 (Arg827Leu) in the affected members of the 1CRD family. Sequencing of the
4CRD family showed a homozygous G to T substitution in exon 13 (Ala547Ser) in \textit{RPGRIp1} gene. Both these novel mutations were observed to segregate in the families as disease causing mutations. None of the mutations were found in the normal, ethnically matched control population. Identification of novel mutations in the \textit{RPGRIp1} gene causing cone-rod dystrophy (a relatively milder condition then LCA) describes the allelic heterogeneity of the \textit{RPGRIp1} gene.

Retinitis pigmentosa GTPase interacting protein 1 (\textit{RPGRIp1}) colocalizes with RPGR to the connecting cilium of the inner and outer segments of the photoreceptors. The RPGRIp1 protein consists of a coiled-coil (CC) domain (homologous to proteins involved in vesicular trafficking) and a C-terminal RPGR interacting domain (RID). The latter interacts with the RPGR protein, providing anchorage to the RPGR in the connecting cilium and participates in protein trafficking through its CC domain and/or in disc morphogenesis (Zhao \textit{et al}, 2003). Photoreceptor connecting cilium is a modified primary cilium that forms a connecting bridge between the inner and outer segments of the photoreceptor cells (Besharse \textit{et al}, 2003). This connecting cilium is a microtubule-based axoneme that originates from the basal body in the inner segment and continues into the outer segment (Liu \textit{et al}, 2004). It provides a critical junction for the transport of phototransduction proteins from inner to the outer segment. (Arshavsky, 2003). It is also thought to be involved in disc morphogenesis. New discs originate from the evagination of the plasma membrane at the distal connecting cilia (Steinberg \textit{et al}, 1980). RPGR protein found in the connecting cilium is necessary for the stability and maintenance of microtubules and
RPGRIP1 provides anchorage to the RPGR protein in the cilia through its RPGR interacting domain (RID).

Three families (442RP, 452RP and 336RP) suffering from arRP were analyzed. Families 442RP and 452RP suffered from early onset RP. The age of onset in both the families was 12-15 years, while the age of onset in 336RP family was 17-18 years. Upon screening, none of the family showed linkage to any known arRP locus. Upon screening with markers of the adRP loci, all three families showed significant linkage with markers D8S285 and D8S1815. This locus contains the RP1 gene that is known to be associated with adRP (Bowne et al, 1999; Pierce et al, 1999; Payne et al, 2000). The association of RP1 gene mutations has been reported in the aetiology of adRP, but never with arRP. Mutation screening of all the three families as well as a large panel of patients was carried out. As most of the reported mutations are clustered in exon 4 (Payne et al, 2000), the 5' region of exon 4 was screened. Sequencing analysis revealed that all the patients of 442RP and 452RP families had a homozygous C to T substitution at nucleotide 1118. This mutation changed the amino acid threonine (ACA) with an isoleucine (ATA) at codon 373. This same change was previously reported to cause adRP in a heterozygous condition (Payne et al, 2000). In another study, Berson et al (2001) categorised this mutation as nonpathogenic because it did not cosegregate with the disease phenotype in one of their adRP families. However, mutation in the 442RP and 452RP families cosegregate with the disease phenotype and all the parents were heterozygous for the same mutation. Upon clinical analysis of the parents, no sign of retinal degeneration was observed.
These observations are convincing evidence to suggest for the first time that this change is responsible for the disease in the recessive Pakistani families.

The results of sequencing the 336RP family revealed a 4bp insertion (1461-1465insTGAA) in exon 4 and was found to be present in a homozygous state in all the patients. This insertion produced a stop codon (TGA) immediately after codon 1461, resulting in the synthesis of a truncated protein of 487 amino acids instead of 2156 amino acids. The parents of the patients and some of the normal individuals were found to be heterozygous for this insertion. Subsequently a panel of 150 patients was also screened for the mutation in the RP1 gene. A heterozygous G to A substitution was found in exon 4 in one patient. This mutation replaced alanine (GCC) with a threonine (ACC) at codon 699. This mutation was not found in any of the 100 normal individuals.

Mutations in the RP1 gene are among the most common cause of RP in many populations and they are responsible for 6-10% of the total RP cases (Guillonneau et al, 1999; Pierce et al, 1999; Sullivan et al, 1999; Liu et al, 2002). The most prevalent mutations are R677X and Q679X (Guillonneau et al, 1999; Pierce et al, 1999; Sullivan et al, 1999; Liu et al, 2002). However, none of these mutations were found in the Japanese population (Kawamura et al, 2004) nor in our Pakistani population. Recently RP1 is reported to be a member of doublecortin (DCX) protein superfamily that serve as protein interaction platforms. Doublecortins are classical microtubule associated proteins (MAP) and are thought to mediate signal transduction pathways by assisting in microtubule polymerization and interaction with the JNK (c-Jun N-terminal kinase) scaffold protein (Reiner et al, 2006).
To find the disease causing mutations in the human SEMA4A gene, a large panel of patients suffering from various retinal degenerative diseases was collected. The panel consisted of 135 RP patients, 25 CRD patients and 30 patients suffering from LCA. SSCP analysis was carried out with the whole panel and samples showing polymorphisms were selected for sequencing. Many variant bands were found that revealed many pathogenic as well as nonpathogenic changes. Sequencing of one of the variant bands found in exon 10 revealed two heterozygous changes. These changes were p.D345H and p.F350C. They are found in 4 patients (RODS002, RODS006, RODS067, RODS119) in a heterozygous state. Of these, two patients were suffering from RP while other two had CRD. Both the mutations were found to segregate together in all the patients. Segregation analysis in one patient's family revealed that he inherited each of the mutations from each of the two parents. Upon clinical diagnosis, both the parents appeared to be normal, while the patient had typical features of CRD. Another disease causing mutation was identified in three RP and one LCA patient. This heterozygous G to A mutation was found to replace the codon of arginine (CGG) with glutamine (CAG) in the patients and was not found in the normal population.

A study on a mouse model showed that dysfunction of the Sema4a gene causes severe retinal degeneration including attenuated retinal blood vessels and depigmentation (Rice et al, 2004). It was also observed that developmental initiation of the photoreceptor cells was normal in the mouse lacking Sema4a but their elongation process is disrupted and the cells did not develop into long organized stacks of discs. Hence, It was suggested that Sema4a provides cell-
cell communication between the RPE and the developing photoreceptor system and transduces an organizational signal for the developing photoreceptor outer segments (Rice et al, 2004).

The identification of novel disease causing mutations in the SEMA4A gene described here is the first report of the involvement of this gene in the pathology of human retinal degeneration.
7.1 CURRENT AND FUTURE THERAPEUTIC APPROACHES:

All types of retinal degenerations cause irreversible damage to vision. Though at the moment there is no cure or prevention of vision loss in humans, there are many aspects of disease pathology where current techniques of molecular biology and genetic engineering are expected to give miraculous results. According to current attempts in the field of therapy, the treatment of most of the cases of RP is not too far. However, there are certain mechanisms that need to be elucidated and many hindrances need to be overcome before any therapeutic intervention can lead to successful strategies in curing human genetic disorders.

Many different studies are underway world wide in describing the mechanisms that lead to pathogenicity of retinal dysfunction and their cure. Animal models of retinal degenerations are very effective in providing an understanding of the mechanism resulting in photoreceptor cell death and to the ultimate cure of genetic disorders. Gene therapy (Farrar, 2002; Dejneka et al, 2003); pharmacological manipulation such as vitamin ‘A’ supplementation (Berson et al, 1993a, 1993b) and growth factors administration (Frasson et al, 1999; Chaum, 2003); identification of cone viability factors (Leveillard et al, 2004); transplantation (Lund et al, 2003) and embryonic stem cell therapy (Ahmad, 2001) are some major approaches that have potential in curing human hereditary diseases. One of the recent advancements in disease genetics studies is the exploitation of RNA interference in identifying gene function and mutation-dependent disease mechanisms (Cheng et al, 2003, Cashman et al, 2005).
The first report of the successful restoration of visual function was in a canine model of human retinopathy. Acland et al (2001) showed that gene therapy using adeno-associated virus (AAV) vector carrying wild type RPE65 gene restored visual function in a dog that was suffering from early and severe visual impairment like human LCA.

Supplementation of vitamin A (9-cis-retinal) for six months in Rpe65-/- mice was shown to delay photoreceptor cell loss and recovery of visual function (van Hooser et al, 2002). Retinal cell transplantation in RP patients is another approach for the repair of retinal function (Radtke et al, 2002). Reduction in photoreceptor cell death has also been achieved by the intraocular gene transfer carrying a growth factor like the ciliary neurotrophic factor (CNTF; Cayouette et al, 1998; Tao et al, 2002). Gene replacement therapy in Rpgrip1 lacking mice by adeno-associated virus (AAV) showed restoration of Rpgrip1 function (to anchor Rpgr in connecting cilia) and preservation of photoreceptor outersegment (Pawlyk et al, 2005).

Embryonic stem (ES) cells have been shown to have potential in the treatment of degenerative ocular diseases. Transplantation of differentiated cells derived from ES cells is being used for the regeneration of lens cells, retinal neurons and retinal pigment epithelial cells (Haruta, 2005). Treatment using bone-marrow derived hematopoietic stem cells were shown to have a positive effect in preventing the loss of cone cells in rd1 (retinal degeneration 1) and rd10 (retinal degeneration 10) mice (Smith, 2004). In another attempt, neural precursor mouse ES cells were administered intra-vitreally in early retinal degeneration and slowly progressing retinal degeneration mouse models. The
results showed that a fraction of the ES cells integrated into the retina (Meyer et al, 2005).

Initial experimental results have proved to be promising, but the above mentioned approaches need a process of trail and re-trial before researchers come to the stage of successful gene therapy in human subjects in the clinics. Identification of all the disease causing mutations; efficient and precise delivery of gene therapy vectors to the appropriate target cell or tissue and long term expression of the delivered gene are some of the challenges that are to be faced by the researchers and therapists.

In conclusion the findings reported in this thesis have significant clinical relevance. The identification of novel genes causing retinal dystrophies leads to further understanding of the biochemical pathways implicated in the process of vision.

The work reported in this thesis will help in the diagnosis of retinal dystrophies at the molecular level. All the mutations found in this study are novel and are specific to Pakistani populations. This has a potential pharmacological and therapeutic significance because in the future, when gene therapy becomes possible, it would assist ophthalmologists to treat these eye disorders that are specifically found in the Pakistani populations.
7.2 REFERENCES:


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