Linkage Analysis of Pakistani Families with Night Blindness

by

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A song of ascents.

[1] I lift up my eyes to the mountains—
where does my help come from?
[2] My help comes from the LORD,
the Maker of heaven and earth.

[3] He will not let your foot slip—
he who watches over you will not slumber;
[4] indeed, he who watches over Israel
will neither slumber nor sleep.

[5] The LORD watches over you—
the LORD is your shade at your right hand;
[6] the sun will not harm you by day,
nor the moon by night.

[7] The LORD will keep you from all harm—
he will watch over your life;
[8] the LORD will watch over your coming and going
both now and forevermore.

Psalm 121

Holy Bible.

*Dedicated to my **Mother***
Words are Worthless
for explication of
Her
LOVE!!

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APS	Ammonium Persulphate
CABP4	Ca^{2+}-Pinding Protein 4
Cav 1.4	Calcium(v) 1.4 L-Type Ca+ Channel
CACNAF1	α1-subunit of an L-type Ca+ Channel.
cM	Centi Morgan
CD	Cone Dystrophy
CSNB	Congenital Stationary Night Blindness
CSNB1	Congenital Stationary Night Blindness Type 1
CSNB2	Congenital Stationary Night Blindness Type 2
CCDC66	Coiled-coil domain containing 66
cGMP	cyclic GMP
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphate
DBCs	Depolarizing Bipolar Cells
EDTA	Ethylene-Diamine-Tetra Acetate
ERG	Electroretinogram
FA	Fundus Albipunctatus
GRK1	G-Proteindependent Receptor Kinase 1
GRM6	Metabotropic Glutamate Receptor 6

GAFa	GAP Domains
GAFb	GAP Domains
GNAT1	Guanine Nucleotide Binding Protein (G protein), Alpha Transducing Activity Polypeptide 1
GPI	Glycosyl Phosphatidylinositol
HBD	Homozygosity By Descent
ipRGCs	Intrinsically Photosensitive Ganglion Cells
ISCEV	International Society of Clinical Electrophysiology of Vision
INL	Inner Nuclear Layer
Kb	Kilo Base
KCl	Potassium Chloride
LOD	Linkage of Odd
L-VDCCs	L-type Voltage-Dependent Calcium Channels
MgCl2	Magnesium Chloride
MLSN1	Melastatin 1
µg	Microgram
µL	Microlitre
mA	Milliampere
Mg	Milligram

mL	Millilitre
mM	Millimolar
Mm	Millimeter
M	Molar
mfERG	Multifocal ERG
NCBI	National Center for Biotechnology Information
Ng	Nanogram
pH	Negative Log of Hydrogen Ion Concentration
NYX	Nyctalopin
ORF	Open reading frame
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PDE	Phosphodiestrase Enzyme
PDE6β	Phosphodiesterase 6β
PDE6γ	Phosphodiesterase enzyme 6 γ
RPE	Retinal pigment epithelium
RDH5	RPE 11-cis retinol dehydrogenase
RP	Retinitis Pigmentosa
RBP3	Retinoid- binding protein 3

Rpm	Revolution per Minute
ROS	Rod outer segments
SAG	S-antigen, gene or Arrestin
SDS	Sodium Dodecyl Sulphate
NaCl	Sodium Chloride
SLC24A1	Solute Carrier Family 24 (sodium/potassium/calcium exchanger), member 1
TEMED	Tetra Methyl Ethylene Diamine
Taq	Thermus Aquaticus
TBE	Tris Borate Ethylene-Diamine-Tetra Acetate
TE	Tris Ethylene-Diamine-Tetra Acetate
TRPM1	Transient receptor potential cation channel, subfamily M, member 1
TRP	Transient receptor potential
U	Unit
UCSC	University of California Santa Cruz
VA	Visual acuity
VAD	Vitamin A deficiency
v/v	Volume by Volume
WHO	World Health Organization

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Mehwish Siddique.

ABSTRACT

Sense of vision is associated with the eye which has incredibly complex structure. Retina is the most important part of eye, which is mainly associated with processes like phototransduction and regeneration of visual pigments. Retinal diseases involving hereditary ones and also blindness represent the dysfunction or degeneration of the retina exhibiting profound visual impairments. Congenital stationary night blindness (CSNB) is also a hereditary dysfunction of retina in which the pathway of visual cascade associated with the rod cells become defective resulting in the loss of night vision. Patterns of inheritance followed by CSNB which is a rare and non progressive retinal condition are autosomal dominant, autosomal recessive or X-linked. The seven candidate genes *(SAG, CCDC66, GRM6, CABP4, RDH5, TRPM1 and SLC24A1)* reported yet for CSNB are involved in visual cascade.

In the current study two Pakistani families (A and B) from the lower areas of Sindh province were investigated. These families were multigenerational, had consanguineous marriages and affected individuals presented symptoms of autosomal recessive CSNB. Linkage analysis by homozygosity mapping was employed for identification of candidate genes in both families by genotyping the available family members with microsatellite markers flanking already known genes for CSNB. The data from the genotyping of loci was further analyzed by using easyLINKAGE plus version 5.02 and a non signficant value of LOD score was obtained for both families confirming the exclusion of linkage to already known genes, thus suggesting the involvement of novel gene/loci in both families.

The study proposes further research for better understanding and exploring of novel genes responsible for CSNB in family A and B by the help of whole genome scan through microsatellite markers or SNP markers.

INTRODUCTION

Blindness, a visual impairment and among the seven categories of increasing disability, is grouped as the sixth one. It can be partial or complete depending upon whether a part of vision is lost or having no perception at all (Frick *et al*., 2003). By the end of 2011, worldwide 285 million people suffered from visual problems out of which 39 million were blind and 246 had low vision. In 2020 the number of blind individuals around the globe would reach up to 76 million that was only 4 million in the year 2000 (Frick *et al*., 2003). World Health Organization (WHO) has stated the major reasons for the onset of blindness to be cataract (an obstruction in the pathway of light due to clouding on the lens), uncorrected refractive errors (far-sightedness or astigmatism near-sightedness), glaucoma (injury of the optic nerve because of a group of diseases) and age-related macular degeneration (the loss of a person's central field of vision). Blindness in children can be due to vitamin A deficiency (VAD) as it is important for the normal functioning of the eye. Environmental and genetic factors both can contribute in the pathogenesis of these disorders (Klein and Francis, 2003).

Remarkable progress is made in finding the genetics of congenital diseases through the advancements in the field of molecular biology in the last years (Dean, 2003). Blindness has remained a major problem in every age of history and inherited blindness is one of the biggest genetic disorders. A number of changes at genetic level have been detected responsible for the inherited eye diseases but still a lot of work has to be done (Daiger, 2004).

The eye is one of the vital organs in the body which transmits details of objects in the environment (Pasternack, 2005). It is composed of incredibly complicated structures and compartments having diversity in their function and origin (Arendt, 2003; Lamb, 2011). In fact it is regarded as a complex optical system, for collection of light, its regulation through a diaphragm, focusing via an adjustable assembly of lenses to the formation of an image and converting this image into a set of electrical signals, and transmission of these signals to brain by the help of complex neural pathways. Retina is one of the most important parts of the eye, and it consists of five types of neural cells including photoreceptors, bipolar cells, ganglion cells, horizontal cells and amacrine cells in addition to these, glial cells called muller cells are also present.

1.1 Photorecptors:

The photoreceptors of the eye make up to 70% of the total complement of human sensory receptors. They get polarized and are thus specific for arresting the photon of light. Two types of vision system are present among mammals depending upon the rods and cones cells of photoreceptors (Pasternack, 2005). Rods are slim, elongated cells in the peripheral region of retina whose outer segments are made of rhodopsin stacked together giving it a rod like appearance and are responsible for scotopic vision associated with the night. Rods have an interior potential of only -30mV in darkness which hyperpolarizes in light to -60mV. Loss of functional short wavelength opsin in most of the nocturnal primates, carnivores and rodents has rendered them to depend upon rods for boosting of sensitivities to vision for dim-light rather than to discrimination of colors (Jacobs *et al.,* 1992; Peichl and Pohl, 2000; Hunt *et al*., 2009). So damage or missing of rods can lead to reduced side vision, impaired night vision or even can cause night blindness.

Cone cells are conical shaped and are present in retina and fovea. Cone cells function to discriminate colors in day time which is dominant vision associated with macula where most of the cones are concentrated. It occurs by the help of three types of cone cells which are S-cone, M-cone and L-cone depending upon the reception of wavelength (Foster *et al.,* 1991). Structurally photoreceptor cells are made up of four parts i.e., the outer segment (OS) which consists of series of disc like structures containing enzymes and proteins for capturing light and initiating photo transduction. Inner segment (IS) containing microsomes synthesizing proteins and enzymes for proper functioning of the cells and nuclear area (N) containing genetic code and synaptic body for the transmission of electrical signals is also present (Bowmaker and Dartnall, 1980).

1.1.2 Signaling Pathways of Rod Photoreceptors:

Rod photoreceptors show response to different light intensities by three signaling pathways which are primary, secondary and tertiary (Zeitz *et al.,* 2005). At low scotopic intensities the primary rod pathway which is from rod cells to rod ON-BC and then to AII amacrine cell, dominates. The excitatory input to all AII amacrine cells is provided to rods by the release of glutamate onto rod ON bipolar cells that are electrically coupled to cone ON bipolar cells consequently inhibiting cone OFF

bipolar cells. This pathway of signaling is completely dependent on mGluR6 signaling cascade and when this gets defective; it causes night blindness (Sharpe *et al.,* 1989; Stockman *et al.,* 1995). At high scotopic intensities signaling followed is from rod to cone cells that is the secondary pathway. The signals from rods into cones flow by the help of electrical coupling between these cells aiding in release of glutamate into the cone ON and OFF bipolar cells. The direct glutamatergic input from rods to cone OFF bipolar cell is the tertiary pathway (Zeitz *et al.,* 2005).

1.1.3 Physiology of Vision:

Cells of the retina are excellently arranged in different strata which are so much helpful for transmission of the phototransduction cascade that is initiated in photoreceptors to second and third order neurons, before it is transmitted through the optic nerve (Aldamesh *et al.,* 2010). The visual cycle occurs in pigment epithelium located at the back of photoreceptors (McBee *et al.,* 2001; Lamb *et al.,* 2004; Travis *et al.,* 2007). Differences in energy of the light bringing successive changes in neurotransmitter output are functionally encoded by rod and cone photoreceptors of the eye. In first step the rod and cone photoreceptors provide input to rod and cone bipolar cells which diverge it into further on and off pathways and this divergence depends upon postsynaptic glutamate receptors. The signaling through the primary rod and cone pathways mainly depend upon glutamate receptors (Maddox *et al.,* 2008)

Vision starts when visual pigment molecules in the outer segment of rod and cone photoreceptor cells absorb photon and become activated. The signals from these activated pigment molecules are sent to G proteins which activates photodiesterase enzyme (PDE) to break down aqueous cyclic GMP (cGMP). The cGMP released from plasma ion channels maintains inward flowing cation current that keeps visual cells depolarized in darkness (Chabre and Deterre, 1989). Depolarization of the photoreceptors is caused by a sudden decrease in light (Sieving, 1993). By the dint of this, release of neurotransmitter from synaptic terminals of photoreceptor cells depolarization takes place. The neurotransmitters then send signal to other cells of retina (Chabre and Deterre, 1989).

1.1.4 Role of Rod cells in Scotopic Vision:

The dim flashes are source of photoisomerization of a single rod among every 10,000 rods is detected by human eye which reveals the exteme sensitivity of night vision (scotopic vision) (Sakitt, 1972, Rodieck, 1998, Ruseckaite *et al.,* 2011). Various defects in the vitamin A cycle result in a reduced rate of rhodopsin regeneration which consequently leads to night blindness. Beginning of this cycle is marked by the photoisomerization of 11-cis retinal to all-trans retinal that is bound to rhodopsin. After passing through a number of transitory intermediates of rhodopsin, release of all trans retinal which are reduced to all-trans retinol dehydrogenase takes place (Haeseleer *et al.,* 1998). This reaction is rate-limiting step in the visual cycle (Saari *et al.,* 1998). The esterification of all-trans retinol with long chain fatty acids (Saari *et al.,* 1988), in conjunction with interphotoreceptor retinoid- binding protein (RBP3) occurs in RPE (Retinal pigment epithelium) which is considerd to be the substrate for an isomerase that converts all-trans retinal ester to 11-cis retinol (Rando *et al.,* 1991). In RPE 11-cis retinol dehydrogenase (RDH5) then oxidizes the 11-cis retinol to 11-cis retinal (Lion *et al.,* 1975). In order to regenerate rhodopsin, 11-cis retinal then returns to the outer segments. For the regeneration of rhodopsin 11-cis retinal is released from RPE (Retinal pigment epithelium) which is thought to be promoted by RBP3 which aids its transfer through inter photoreceptor matrix (Pepperberg *et al.,* 1993; Gonzalez-Fernandez *et al*., 1998). The delayed dark-adaptation or night blindness can result from any failing in the occurrence of above reactions.

1.2 Diseases related to Retina:

Alterations in the genetic makeup have a significant impact on the retina (Jelcrick *et al.,* 2011). Many of the diseases are associated with degeneration as well as with the dysfunction of retina including Leber Congenital Amaurosis, retinitis pigmentosa (RP), congenital stationary night blindness (CSNB), cone or cone-rod dystrophy, macular degeneration, color blindness, myopia, chorioretinal atrophy and retinoschisis (Zhang *et al.,* 2005).

1.2.1 Retinitis Pigmentosa (RP) in relation to the Night blindness:

RP can be related to night blindness as it also is characterized with night blindness, attenuation of retinal vessels especially the arteries, typical fundus changes including a waxy-disc appearance, gradual constriction of visual fields (Boughman, 1980),

which can increase with age and bone-spicule pigmentation in the midperipheral retina and finally loss of central vision. The rod ERG response is either totally lost or is reduced while the cone response is normal and in some cases may become undetectable (Heckenlively *et al.*, 1988). Mutation in genes which cause RP also can cause night blindness e.g arrestin 1147delA is a frequent reason of Oguchi disease (Nakazawa *et al.*, 1998).

1.3 Congenital Stationary Night blindness:

Congenital stationary night blindness (CSNB) is a genetically determined rare, group of disorders. This is basically a functional defect which makes it much different from other retinal dystrophies (Szabo *et al.*, 2007) as CSNB is caused by flaw in the signaling from photoreceptor to bipolar cells (Zeitz *et al*., 2007, 2009). In this disorder there is an enduring scarce vision in the dark, usually having nystagamus and myopia (Rebhun *et al.*, 1984; Sandmeyer *et al.*, 2007). Despite of having a normal fundus appearance, CSNB is categorized into two subgroups based on electroretinographic (ERG) and psychophysical findings: complete (cCSNB) and incomplete (icCSNB) (Zeitz *et al*., 2007, 2009).

1.3.1 Diagonistics:

The function of the photoreceptors and depolorizing bipolar cells can be investigated by electroretinograph (ERG) and protocols for this method are published by The International Society of Clinical Electrophysiology of Vision (ISCEV) (Marmor *et al.*, 2004).

1.3.1.2 Electroretinography:

This method is used for measurement of objective electrical responses of various cells present in retina inclusively photoreceptors, inner retinal cell and gangalion cells and is even sensitive to mild visual impairments. For this purpose electrodes are placed near cornea and skin of eye which on exposure to standard stimuli depicts the signal showing the course of signal's amplitude (voltage) time (Marmor *et al.*, 2004). Dark adapted eye when undergo through flash ERG represents the response of rods. ERGs contain an a-wave (initial negative deflection) which is response of photoreceptors when they are hyperpolarized followed by a b-wave (positive deflection) produced by a mixture of muller cells and photoreceptors bipolar amacrine (Hood *et al.*, 1994; Robson & Frishman, 1998; Xu *et al.*, 2003).

There are two types of full field and multifocal ERGs (mfERG). Full field represents the total activity of retina in response to a light flash and the multifocal ERG (mfERG) shows retinal dysfunction in small areas (Marmor *et al.,* 2003). Two types of CSNB are categorized on the basis of electroretinography which are Schubert Bornschein and Riggs (Schubert and Bornschein 1952; Riggs 1954; Kabanarou *et al.,* 2004). Schubert Bornschein has an electronegative ERG response at the scotopic bright flash in which b-wave is smaller than a-wave. Decreased visual acuity, myopia, and nystagmus can be associated with it. It is depicted by X-linked and autosomal recessive CSNB (Kabanarou *et al.,* 2004; Riazuddin *et al.*, 2010). Riggs type of CSNB is distinguished by proportionally reduced both a-wave and b-wave and visual acuity is within a normal range; no symptoms of myopia and/or nystagmus are present (Riggs, 1954; Riazuddin *et al.*, 2010).

1.3.1.3 Funduscopy:

It is a diagnostic procedure to examine the fundus of eye including retina using an instrument called funduscope. Fundus consists of the blood vessels to feed the eye, retina, the optic disk, and the choroid. Abnormalities of blood vessel, hemorrhages, pigmentation, exudates and cotton wool spots can be detected by funduscopy (Crick and Khaw, 2003). Three different forms of this procedure are known including direct, indirect and slit-lamp fundoscopy. However, frequently the fundus examination is normal but appearance with tilted optic disks and myopia is frequent in the X-linked and autosomal recessive forms of CSNB (Dryja, 2000).

1.4 Variants of CSNB:

Oguchi disease and Fundus Albipunctatus (FA) are the two variants of CSNB which display an abnormal fundus appearance.

1.4.1 Oguchi Disease:

Autosomal form of CSNB found rarely, having abnormal slow dark adaptation after light exposure and fundus discoloration along with abnormalities of ERG (Fuchs, 1995). The significant reduction in a-wave that is mixed rod–cone ERGs elicited by a bright flash while the b-wave is absent. It was documented by Oguchi in 1907 as a variant of CSNB and was later characterized phenotypically by Mizuo in 1913. Mizuo-Nakamura phenomenon, a typical sign of Oguchi disease is very common among Japense population (Fuchs, 1995). Mutations in genes *GRK1*and *SAG* result in

Oguchi disease of two types, Oguchi disease-1 and Oguchi disease-2 respectively (Yamamoto *et al.,* 1997; Hayashi *et al.,* 2007). A variant form of Oguchi disease is identified by novel c.827+623_883del mutation in *GRK1* accompanied by much phenotypic variability (Zhang *et al.,* 2005). Evidences for co-existence of Oguchi and RP in the same family or in same individual are also present. In such cases the clinical characteristics of Oguchi may get masked and diagnosis becomes difficult (Yamanaka, 1969; Sonoyama *et al.,* 2011).

1.4.2 Fundus Albipunctatus (FA):

A recessive retinopathy characterized by night blindness from early childhood, delayed dark adaptation because of defect in both rod and cone pathways and prominent fundus abnormalities. Patients having FA exhibit numerous white-yellowish small, round or elliptical discrete subretinal spots or lesions basically in retinal pigment epithelium. These spots are scattered in the mid periphery and perifovea (Sekiya *et al.,* 2003; Yamamoto *et al.,* 2003). It was reported for the first time to be caused by significant changes in 11-*cis* retinol dehydrogenase *RDH5* gene in 1999, since then many frame shift, missense, and inframe mutations have been identified (Sekiya *et al.,* 2003; Yamamoto *et al.,* 2003). Vitamin A deficiency and abetalipoproteinemia leaded to concept of defect in vitamin A or its metabolism (Fernandez *et al* 1999) but this notion was refuted as its supplementation yielded no positive results. Some of the patients suffering from FA can also develop cone dystrophy (CD) or macular degeneration (MD) with progressive loss of visual acuity by way of increasing age but a young boy of 9 years having compound heterozygous mutations of Tyr281His and Leu310GluVal in *RDH5* gene exhibited reduced visual acuity and macular degeneration (Miyake *et al.,* 1992; Nakamura *et al.,* 2000; 2002; 2003). Twin sisters (23 years) also suffered from CD and had combination of compound heterozygous mutation of Val132Met and Arg280His in *RDH5* (Nakamura *et al.,* 2004). Eleven mutations for this gene causing FA, nine of these were novel, and are reported by (Sergouniotis *et al.,* 2011) with patients having phenotypic variability.

1.5 Mode of inheritance of CSNB:

Description of all manners of Mendelian inheritance have been documented for this disorder, thus it can be categorized into autosomal recessive, autosomal dominant and X-linked types of congenital stationary night blindness (Gal *et al.,* 1994). These forms

of CSNB also have fundus with normal appearance. The genes associated are involved in either phototransduction cascade or with transmission of signals from photoreceptor to adjacent bipolar cells (Zeitz *et al*., 2008).

1.5.1 Autosomal Recessive CSNB:

It is a rare form of phenotype which is not well characterized. Clinical presentation includes reduced visual acuity, myopia, and nystagmus having normal fundus. CSNB inherited in this manner occurs because of the mutations in following genes *GRM6, TRPM1, SAG, CABP4, GRK1, SLC24A1, RDH5* and *CDCC66*. These genes play major role downstream of phototransduction cascade in transmission of signals (Zeitz *et al*., 2008). Description of these genes including their function and mutations is given below.

1.5.1.1 *GRM6:*

Metabotropic glutamate receptor 6 (mGluR6) is encoded by *GRM6*, mapped to 5q35. A single isoform is expressed in the dendrites of rods and cones (Vardi, 2000). mGluR6 belongs to a G-protein-coupled receptor family containing eight members which are divided into three groups I, II and III based on their pharmacologic properties, homology, and putative signal transduction mechanisms, (Nakanishi, 1994; Pin and Duvoisnin, 1995; Gerber, 2003). It contains 10 exons and encodes an 877 amino acid protein made of extracellular terminal domain which is large bi-lobed consisting of glutamate binding site, an intracellular COOH-terminal domain and seven G protein coupled receptor (GPCR) transmembrane domains (Zeitz *et al*., 2007). CSNB is associated with mutations in *GRM6* express myopia as well (Xu *et al.,* 2009). Sequence variations including missense, frameshift and nonsense mutations are identified which results in abolition of signaling and trafficking within the retina resulting in diseased phenotype (Dryja *et al.,* 2005, Zeitz *et al.,* 2005). Mostly missense mutations in the ligand binding domain, cystein rich domain and transmembrane domain occur among amino acid sequences which are conserved in all the groups of GluRs protein of group III, associated with CSNB are mainly because of retention of protein intracellularly in ER (Zeitz *et al*, 2007).

1.5.1.2 *TRPM1:*

Transient receptor potential channels (TRP) are non selective cation channels and were discovered 40 years ago by the characterization of a spontaneous mutation in

Drosophila (Phillips *et al.,* 1992). These channels are mostly expressed in ON bipolar cells in association with photopigment melanopsin in the subset of intrinsically photosensitive ganglion cells (ipRGCs) which are engaged in non-image-forming visual and TRP channels take part in photo transduction cascade (Warren *et al*., 2006; Lamb *et al.,* 2007; Audo *et al.,* 2009). Functionally they allow entrance of Ca^{+} in hyperpolarized cells for the initiation of phosphatidylinositol signaling pathways and protein kinase C triggering interacelluar responses (Clapham *et al.,* 2001).

TRPM1, founding member of the subfamily M of TRP channels was identified in mouse having human homologs localized on rod ON bipolar cell dendrites (van Genderen *et al.,* 2009; Koike *et al.,* 2010). Alternative splicing results in the production of two forms trpm1-L and trpm1-S in mouse where it shows expression in a variety of tissues while humans posses no such distinction (Audo *et al.,* 2009). It functions as a cation channel in retinal ON-bipolar cells because of its sensitivity and current voltage relationship. TRPM1 is thought to be linked with the depolarization of ON- bipolar cells and mutations in this gene leads to loss of function of ON-bipolar cells which causes onset of CSNB in mice and Appaloosa horses (Audo *et al.,* 2009). Five mutations were identified in Japense patients by Nakamura *et al*., (2010) which produced either abnormal protein or resulted in the mislocalization of TRPM1. Mutations in *TRPM1* include missense, frameshift due to deletion, inversion, substitution and translocation in certain exons which result in the elimination of inward current or in removal of whole channel (Genderen *et al.,* 2009).

1.5.1.3 *CABP4:*

Calcium-binding protein 4*(CABP4)* was mapped on 11q13.2 and encodes a protein consisting of 275 amino acids encoded by six exons of *CABP4* gene (Aldahmesh *et al.,* 2010). It has four, helix loop helix EF-hand motifs structures for coordination of Ca^{+} with ligands (Bentley and Réty, 2000, Haeseleer *et al.,* 2004). Localization of CABP4 in retinal rod and cone photoreceptors synaptic terminals is confirmed through Northern blot analysis (Haeseleer *et al.,* 2004; Zeitz *et al.,* 2006). CABPs are neuronal Ca^{2+}-binding proteins with similarity to calmodulin and modulate voltage-dependent Ca^{2+} channels (Lee *et al.,* 2002). In association with C-terminal domain of Cav1.4, it helps in shifting activation of Cav1.4 to hyperpolarized cells indicating that CABP4 is an important regulator of Ca^{+} influx and transmitter release in photoreceptor synaptic terminals (Haeseleer *et al.,* 2004).

Two different but important mutations (c.800_801delAG and c.370C→T) were reported by Zeitz *et al.,* (2006) in Swiss families having autosomal recessive CSNB. These cause an addition of novel 91 amino acids in protein and produced an altered protein with impairment in the Ca^{2+}-binding affinity and capacity (Zeitz *et al.,* 2006). Photoreceptor synapses were severely interrupted by both functionally and anatomically in CaBP4-deficient mice. Absence of CaBP4 leads to reduction in the glutamate concentration present at bipolar mGluR6 receptors (Haeseleer *et al.,* 2004). Reduction in number of transcripts upto 30 to 40% then normal range is due to mutations occurring in this gene which leads to defective signaling resulting in CSNB (Littink *et al.,* 2009).

1.5.1.4 *RDH5:*

Retinol dehydrogense 5 a stereo specific enzyme (RDH5) encoded by a gene located on 12q13.2 and is a member of short chain dehydrogenose/reductase (SDR) superfamily. It is an integral membrane protein of smooth endoplasmic reticulum (Simon *et al.,* 1995; 1996; Sergouniotis *et al.,* 2011) and is anchored by N and C terminal hydrophoibic segments of peptide. The oxidation and reduction of hydrophobic substrates like retinoids and steroids is catalyzed by SDR superfamily (Simon *et al.,* 1995).

RDH5 is expressed in various tissues in human but in extra ocular tissue expression accounts for 100% (Wang *et al.,* 1999). It accounts for most of the 11-*cis* retinol dehydrogenase activity in retina responsible for oxidation of 11-cis retinol to synthesize 11-cis retinal (Simon *et al.,* 1996). Human RDH5 is a 318 amino acid protein and mutations in this gene are mostly responsible for the rare form of night blindness, fundus albipunctatus because of decline in dehydrogenase activity and stability in vitro (Sekiya *et al.,* 2003; Yamamoto *et al.,* 1999; 2003). Mutations in *RDH5* lead to reduced autofluorescence signal possibly because of absence of retinoid-derived fluorophores (Schatz *et al.,* 2010).

1.5.1.5 *CCDC66:*

Coiled-coil domain containing 66 (*CCDC66*) gene is found on human chromosome 3 with 19 exons spreading over 64.6 kb. This gene is conserved in various vertebrate species and displays a complicated pattern of differential RNA splicing resulting in production of various isoforms in the retina (Dekomien *et al.,* 2009). In man the

anticipated open reading frame (ORF) encodes a 914 aa protein which is expressed in a wide range of tissues. Expression studies show that CCDC66 is found in inner sections of photoreceptors and the ganglion cell layers and external plexiform in man mouse and dog at a depleted level (Dekomien *et al.,* 2009).

The hepta repeat pattern containing at least one coiled-coil domain makes up CCDC66 proteins. They also form a cable-like structure made up of secondary structure composed of two or more α-helices.CCDCC66 were found in alpha keratin for the first time (Burkhard *et al.,* 2001). By the configuration of firm bundles of fibers helical cables perform a mechanical role in proteins (Liu *et al.,* 2007).

1.5.1.6 *GRK1:*

G protein-dependent receptor kinase 1; GRK1 also called RHOK is conserved element oflight adaptation and restoration pathways (Young *et al.,* 2007). It is located on 13q34 consisting of 7 exons and encodes a 563 amino acids (Khani *et al.,* 1996; Zhao *et al.,* 1998). It is a member of guanine nucleotide-binding protein (G protein)-coupled receptor kinase subfamily of the Ser/Thr protein kinase family which deactivates rhodopsin by its phosphorylation. It is found in rods, cones and pinealocytes in humans (Maeda *et al.,* 2003; Hayashi *et al.,* 2007). This family of enzymes has broad and overlapping substrate specificities because of alternative splicing which produces different isoforms (Xinyu *et al.,* 1998). The expression of GRK1 is inhibited by players such as S-modulin/recoverin which interact with aminoacids present at the N-terminal and can inhibit its activity (Torisowa *et al,* 2008).

Conserved transcriptional complex contains Otx2–Crx transcription factors at the core which controls expression of *GRK1* in photoreceptors (Young *et al.,* 2007). In addition to presence of a unique T-rich module interacting with TATA-binding proteins this particular region also consists of a bicoid-type homeodomain recognition cassette. Principally interactions of homeodomain involve Crx and secondarily Otx2 (Simeone *et al.,* 1992; Nishida *et al.,* 2003). The E/P (enhancer or promoter) is strongly stimulated by both of these transcription factors. The persistent E/P activity, in absence of Crx, shifts from the outer retina to the inner to follow the Otx2 pattern (Nishida *et al.,* 2003). Absence of rod transcription factors, Nrl and Nr2e3, do not affect these spatial patterns and the RK transcriptional activity preceded the surge in

rod-specific transcription (Mears *et al.,* 2001; Peng *et al.,* 2005). The basic factors in governing localization of RK, E/P activity in retina and photoreceptors are conserved bicoid homeodomain factors (Young *et al.,* 2007).

1.5.1.7 *SAG:*

S-Antigen *(SAG)* or alternatively S-Arrestin or Rod Arrestin is located on 2q37.1 and codes for a photoreceptor-specific soluble protein implicated in the recovery phase of light transduction (Benovic *et al.,* 1987). It is one of the major proteins of rod outer segment, which has an essential role in quenching phototransduction cascade by inactivation of phosphorylation-activated rhodopsin (Nakazawa *et al.,* 1998). The currently available data illustrates functional members of the arrestin gene family which are four (Freedman and Lefkowitz, 1996; Ferguson, 2001) out of which two visual and cone arrestin exclusively express in retinal cells. They sterically block the interaction of receptor G-protein and are thus desensizatizing those when bound to agonist occupied G- protein coupled receptors (GPCRs) or light activated and those are phosphorylated by GRKs. Microtubules in the inner segment of dark-adapted rod cells may work as a "sink" for binding of arrestin when it is present here (Gurevich, 2006; Nair *et al.,* 2004). Upon exposure of light it translocates to outer segment (Elias *et al.,* 2004). There may be sufficient arrestin in rod for binding to the entire pool of rhodopsin in a completely photo-bleached cell (Strissel *et al.,* 2006).

Structurally arrestin protein consists of N terminal (8-180) and C terminal (188-362) domains which consists of seven stranded β sandwich (Graznin *et al*., 1998; Hirsch *et al*., 1999). The significant part in recognition of light-activated rhodopsin, is played by N terminal domain of the molecule whereas within C terminal domain a secondary-receptor-binding region is present (Gurevich *et al*., 1995). Sensation of phosphate region is completed by an area localized between N and C domains and polar core of the protein is formed by it. When interactions between phosphate sensor region and C-terminal tail are established then arrestin is allowed to bind phosphorylated receptor with high affinity that keeps it in an inactive state these are disrupted upon receptor binding (Hirsch *et al*., 1999).

1.5.1.8 *SLC24A1:*

SLC24A1 (solute carrier family 24 (sodium/potassium/calcium exchanger) member 1) gene is located on chromosome 15q22 and codes for sodium-calcium potassium

exchanger (NCKX). Its mRNA is found in retinal rod photoreceptors particularly in the outer segment but through insitu hybridization it is shown that its signals were also detected in inner segments and ganglion layers (Sharon, 2002). It is responsible for the transport of Ca^+ and also performs Ca, K and Na antiporter activity through NCKX and *SLC24A2* gene. Span of its transcript is 5.7 kb and consists of ten exons that encode a 1099 amino acid protein (Sharon 2002).

Fluctuations in Ca^+ and Na^+ level in outer segments of photoreceptors are important for phototransduction and balance between outflux of Ca^+ and influx of Na^+ is thought to be provided by SLC24A1. Missense mutation (Ile992Thr) in this gene produced poorly functional protein which leaded to CSNB associated with Riggs type of ERG response (Sharon, 2002; Li *et al.*, 2002). The mutation responsible for autosomal CSNB is a 2 bp deletion c.1613_1614del in transmembrane domains of SLC24A1 (Riazuddin *at el.,* 2010). The absence or reduction in this phenomenon consequently causes abnormal level of Ca^+, resulting in disease phenotype (Sharon, 2002; Riazuddin *at el.,* 2010).

1.5.1.9 Mechanism Underlying CSNB:

Information channels which are present postsynaptically on ON bipolar cells responds to increasing and decreasing light intensity (Schiller, *et al.,* 1986) and answers differentially to the release of only excitatory neurotransmitter glutamate (Dieck and Brandstatter, 2006). Light leaves an opposite impact on the polarization of bipolar cells which is due to presence of two distinct type of glutamate receptors present on ON and OFF bipolar cells (Stockman *et al.,* 1995). Mutations in genes which encode components of phototransduction cascade or any abnormality in proteins involved in signaling from photoreceptors to the adjacent second order neurons are responsible for CSNB (Zeitz *et al.,* 2006). A defect of the ON response, which leads to an electronegative combined rod-cone ERG, based on a severely reduced b-wave is the most apparent phenotypical attribute of patients with cCSNB (Miyake *et al.,* 1986; Audo *et al.,* 2009).

In the dark, photoreceptors depolarize increasing rate of glutamate (Figure 1.1) but it reduces with the induction by light which causes hyperpolarization (Tachibana, 1993). Glutamate binds to mGluR6, and activates Gαo1 and Gβ5, subunits of a heterotrimeric G protein they act as second messanger in this pathway (Rao *et al.,*

2007). This in turn by an unidentified mechanism leads to closure of an as-yet-unknown cation channel (Vardi *et al.*, 2000; Dhingra *et al.*, 2002; Zeitz, 2007). The ON response is initiated with shutting down of the G protein cascade upon light exposure as release of glutamate from photoreceptor decreases. Afterwards, ON bipolar cell depolarization gives rise to b-wave with opening of the cation channels. The loss of mGluR6 at the cell surface mostly occurs because of mutations in *GRM6.* Glutamate released from the photoreceptors if not modulated, cannot be correctly sensed by bipolar cells marking the failure of depolarization and so an extremely reduced b-wave is produced (Zeitz *et al*., 2007).

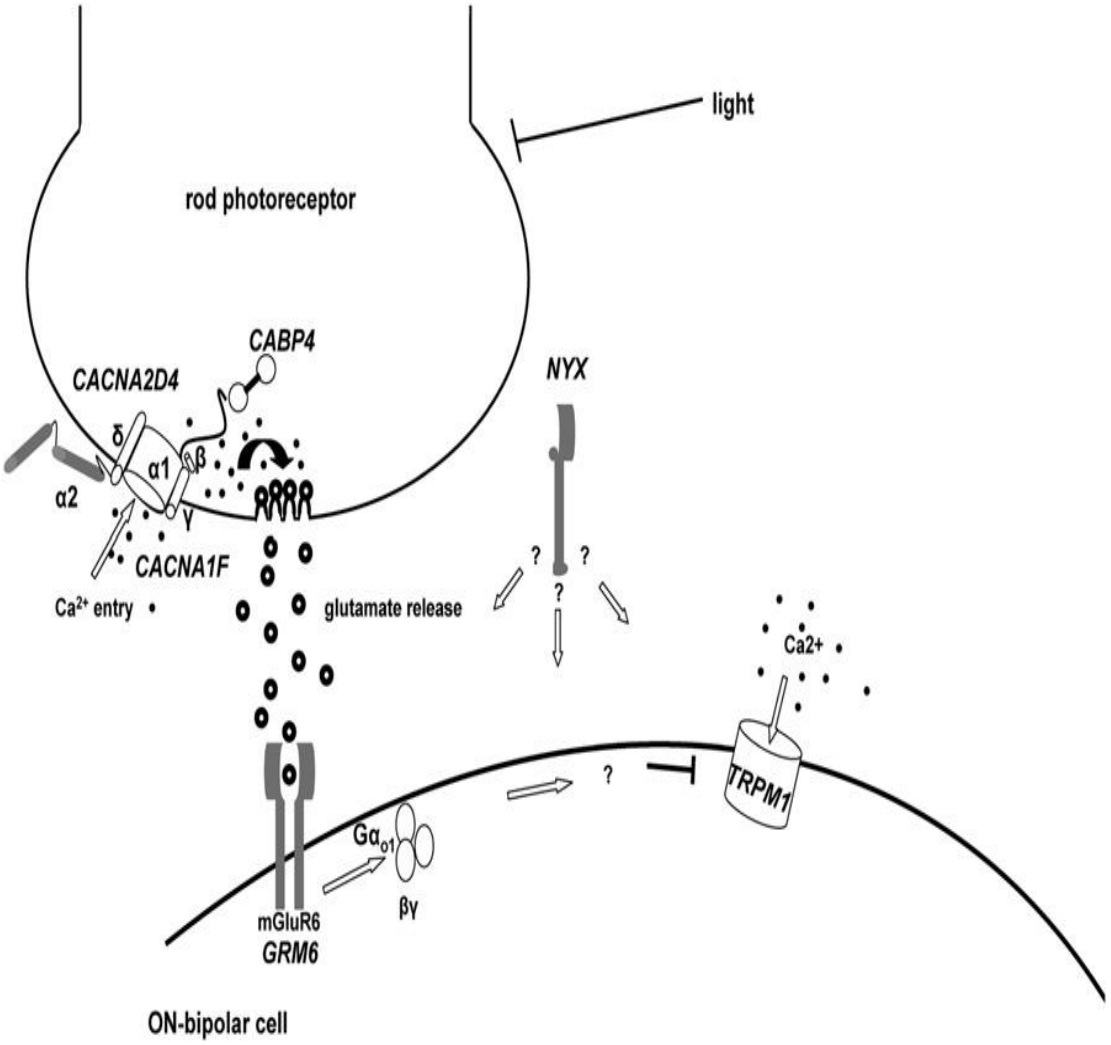

Figure1.1. Representation of proteins involved in signal transmission from photoreceptors to adjacent ON bipolar cells and cause a cCSNB phenotype in humans and/or mice, Adapted from(Audo *et al.,* 2009).

1.5.2 Autosomal Dominant Congenital Stationary Night Blindness:

Large families with autosomal dominant CSNB are present like French Nougaret genealogy first described in 1838, which was reinvestigated in 1907 and also an extended Danish pedigree first published in 1909 by Rambusch and rediscovered in 1999. However mutations in various members of the rod phototransduction cascade (*RHO, GNAT1,* and *PDE6B)* lead to this type of phenotype. As the patients have less chance of myopia and nystagmus properties so mutations cause a nonprogressive and milder type of phenotype (Ruether *et al.,* 1993; Zeitz *et al*., 2008).

1.5.2.1 *RHO:*

RHO located on 3q21-24 codes for the protein; Rhodopsin, which is a member of G-protein, coupled receptor superfamily and is important for normal vision, particularly in low light conditions (Wang *et al*., 1980; Neidhardt *et al*., 2006). It has three domains and functionally is responsible for induction of transduction cascade in the retina. It consists of an apoprotein opsin and a chromophore, 11-cis retinal. The chromophore, 11-cis retinal is covalently bound to opsin via a protonated Schiff base linkage and put a stop to activation of G-protein in the dark. Mutation in *RHO* gene first time reported in 1990 was found to be responsible for RP (Farrar *et al.,* 1990). The photoreceptors proteome analysis indicates predominant levels of rhodopsin within them (Liu *et al.,* 2011).

Autosomal dominant CSNB which is an infrequent retinal dysfunction caused by at least four mutations in the *RHO* gene which are (p.Gly90Asp, p.Thr94Ile, p.Ala292Glu and p.Ala295Val) (Dryja *et al.*, 1993; Rao, 1994; Gross, 2004; Zeitz *et al*., 2008). These mutations results in conformational changes in protein as they mostly influence the activation of transducins and opsins by introducing amino acids which are nonpolar and constrains the rotational freedom on backbone of polypeptide which destabilizes α helices, because they are induced to assume a more limited conformation with unfavorable torsional angles relative to substituted amino acids (Cornish *et al.,* 1994; Lin *et al.,* 1998). Constitutive activation of transducins results from mutations in this gene and permanent activation of rods cannot convey significant signals to the brain in response to light. This loss of signaling causes night blindness in affected individuals (Zeitz *et al*., 2008).

1.5.2.2 *GNAT1:*

Guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1 *(GNAT1)* is located on 3p21. It stimulates the coupling of rhodopsin and cGMP-phoshodiesterase from receptor during visual impulses and is also involved in various transmembrane signaling systems acting as modulators (Slepak *et al*., 1995, Cowan *et al*., 2001, Slep *et al*., 2001). Belonging to one of the three families of GTP binding protein functioning of GNAT1 mainly depends upon intrinsic GTP-driven conformational switch in combination with intrinsic GTPase activity which is called Switch 2 domain (Dryja *et al.*, 1996). Different genes encode the transducin alpha subunits in rods and cones; *GNAT1* encodes the alpha subunit in rods. Expression of this gene is also reported in other cells, and has been concerned with bitter taste transduction in rat taste cells (Szabo *et al.*, 2007). Naugart type CSNB results from a missense mutation at position 37 in *GNAT1* (Dryja *et al.*, 1996). The production of α-transducin which is unable to bind light activated rhodopsin so cannot bind to its downstream effector molecule is because of mutations in this gene which result in adCSNB (Dryja *et al.*, 1996; Szabo *et al.*, 2007).

1.5.2.3 *PDE6B:*

A member of phosphodiesterase family of effector proteins for transducins, is Phosophodiesterase 6, mapped to 4q 16.3 (Gal *et al.*, 1994) and it catalyzes hydrolysis of 3' cyclic phosphate bonds in adenosine selectively and/or guanine 3',5' cyclic monophosphate (cAMP and/or cGMP) and regulate intracellular level of cAMP and cGMP. By controlling the degradation rate of second messengers these proteins regulate their duration of action, cellular levels and localization. Rod photoreceptors absorbs photon that leads to activation of cGMP phosphodiesterase (PDE), which results in rapid hydrolysis of cGMP, closing of cGMP-gated cation channels, and ultimately hyperpolarization of the cell. PDE is made up of alpha, beta, and gamma subunits so it is peripheral membrane heterotrimeric enzyme. The Tandem GAF domains (GAFα and GAFβ), an N-terminal domain of unknown function and a C-terminal catalytic domain together make each PDE6β subunit (Guo *et al*., 2005). GAFα domain is noncatalytic and binds cGMP with high affinity but is differnt from the cGMP hydrolytic site. First mutation in *PDE6β* was reported was by Gal *et al*., (1994) which involves substitution of H258N in a conserved region between two GAF

domains adjoining the documented PDE6γ-interacting domain. Similar mutation was identified by Tsang *et al*., (2007) which resulted in CSNB in mice.

1.5.3 X-Linked Congenital Stationary Night blindness:

Congenital stationary night blindness can follow X-linked mode of inheritance. It can be of two types on the basis of clinical manifestations and genetically CSNB1 and CSNB2 depending upon the mutations in genes *NYX* and *CACNA1F* respectively (Bech-Hansen *et al.*, 1998; Zeitz *et al*., 2003).

1.5.3.1 CSNB1:

It is also termed as complete form of X linked CSNB and is characterized by extreme loss of rod-mediated visual sensitivity, an absent b-wave electroretinogram accompanied by a variation in retinal waves of the immature retina. It is caused by mutation in the gene *NYX* located on Xp11.4 (Zeitz *et al*., 2003; Demas *et al*., 2006) that codes for Nyctalopin, member of small leucine rich proteoglycans that functions in growth control, cell signaling and formation of the extracellular matrix (Bech-Hansen *et al*., 2000; Pusch *et al*., 2000; McEwan *et al*., 2006). It is essential for the expression and function of TRPM1 channel (Bellone *et al.*, 2008). It acts as an adhesion molecule identifying the ON pathway circuitry in the retina (Bech-Hansen *et al.*, 2000; Pusch *et al.*, 2000). Despite of having information of its localization to both cellular and synaptic sites the exact location of expression of nyctalopin in retina is not found and exact function of nyctalopin in synaptic transmission is also yet not known (Bech-Hansen *et al.*, 2000; Bahadori *et al.*, 2003; Pesch *et al.*, 2003; Morgans *et al.*, 2006; Bahadori *et al.*, 2006). Synaptic transmission between photoreceptors and DBCs is the primary function of nyctalopin. Nyctalopin contributes significantly in retinal processing but that is only restricted to postsynaptic side of the photoreceptor to synapse of BC (bipolar cell) (Bech-Hansen *et al.*, 2000; Jacobi *et al.*, 2002).

Structurally it consists of 11 leucine-rich repeats (LRR), an ER signal peptide on N-terminal, and a C-terminal glycosylphosphatidylinositol (GPI) membrane anchor. Mutations in this region are mostly thought to be associated with disease phenotype in humans, flanked by LRRs that are cysteine-rich, motifs having short-sequences and mediate protein–protein interactions (Zeitz *et al.*, 2003). In transgenic mice its expression is mainly observed on DBCs dendrites by EYFP-nyctalopin fusion protein (Gregg *et al.*, 2007). Due to mutations in *NYX*, a postsynaptic deficit from

photoreceptor to communication in bipolar cell results because it is necessary for normal synaptic transmission between retinal photoreceptors and depolarizing bipolar cells (DBCs). It results from the failure of neurotransmitter glutamate release (Gregg *et al.,* 2007). To date greater than 30 mutations in this gene have been ascertained in humans (Bech-Hansen *et al.,* 2000; Pusch *et al.,* 2000; Jacobi *et al.,* 2002; Zeitz *et al.,* 2005).

1.5.3.2 CSNB2:

It is also said to be incomplete form of CSNB. The CSNB2 patients show a typical negative bright-flash ERG which has larger a-waves, but severely reduced b-waves supporting a defect in neurotransmission from retinal photoreceptors to second order neurons and a very abnormal dim scotopic ERG (Tremblay *et al.,* 1995). An indication of normal rod function is present, but that transmission from rods to rod bipolar cells is critically compromised. Rod cells show some adaptability to darkness but cone function also is impaired. Mutation in the voltage gated calcium channel (VGCC) gene results in CSNB2 phenotype. VGCC brings about membrane depolarization of Ca^{2+} dependent intracellular signaling by mediation of Ca^{2+} ion influx or by direct conformational coupling to intracellular Ca^{2+} release channels (Catterall *et al.,* 2005).

CACNA1F is located on Xp11.23 and codes for Cav1.4, a member of L-type Ca^{+} channel (LTCCs), expressed in active zone specifically at the ribbon synapses of photoreceptor and are involved in release of neurotransmitters with changing membrane potentials. An abrupt hyperpolarization of the photoreceptors occurs by absorption of light from depolarized state which prevents the neurotransmitter release. This can be maintained by the rapid activation of Ca^{+} channels at negative voltages and slow inactivation properties present in Cav1.4 α subunits (Koschak *et al.,* 2003; Baumann *et al*., 2004; Peloquin *et al*., 2008). Yet 40 structural changes have been described in Cav 1.4 α1 in CSNB patients, involving truncated α1 subunits, premature stop codon due to splice site mutation leading to nonsense mediated decay (NMD) of RNA. Loss and gain of function mutations in *CACNA1F* in CSNB2 affected males have also been reported by Hoda *et al.*, (2005) which are S229P, G369D, L1068P, and W1440X.

1.6 Pakistani Perspective:

Pakistan is a developing country and is encountering different issues including lack and providence of basic necessities of life to the major extent. Adding more to this, the rate of consanguineous marriages in Pakistan is much greater, thus is suitable for studies of genetic outcomes (Bittles and Black, 2009). These parameters in Pakistan are leading to the prevalence of more genetic disorders. There is a greater influence of consanguinity on the prevalence of disorders which are less common and this generalization is applicable to recessive disorders (Bittles, 2001; Pasternack, 2005). Pakistani population has the highest degree of consanguinity so it is most suitable resource for mapping of recessive loci, according to recent survey retinal diseases almost 57.3% occur in consanguineous families (Adhi and Ahmad, 2002). The incidence of night blindness in Pakistan among women is about 10 percent with high prevalence in Sindh and Khayber Pakhtoon khwa and is more common in individuals over 50 years of age. In Pakistan 9.4 % women are facing the problem of night blindness (Sher, 2004).

Regarding the work on genetically induced night blindness especially CSNB significant work has not yet been done in Pakistan. Only few studies have been carried out on the genetic basis of inherited forms of CSNB in Pakistan.

A variant form of Oguchi disease in a family from the Punjab province of Pakistan having 3 affected and 12 unaffected members was mapped to 13q34 and is associated with mutation in *GRK1* which is supported by linkage data (Zhang *et al.*, 2005). In the same gene *(GRK1)* a novel nonsense mutation (c.614C>A; p.S205X) in exon 1 was reported by Azam et *al.,* (2009). This was a large family from central region of Punjab province which was initially diagnosed with RP, showed a novel mutation in *GRK1* gene associated with Oguchi disease (Azam et *al.,* 2009). In 2010 in a study on Pakistani population a new gene has been identified for autosomal recessive congenital stationary night blindness. The disease interval was localized to chromosome 15q by genome-wide scan and the critical interval refined a 10.41 cM (6.53 Mb) region that elucidated recombination events in diseased individuals. This region housed *SLC24A1*, a member of the solute carrier protein superfamily (Riazuddin *et al.*, 2010). In 2011 a study carried out on fundus albipunctatus, a variant of CSNB on two consanguineous families. The alleles of markers on chromosome 15q flanking *RLBP1* segregated with the disease phenotype. Sequencing of *RLBP1*

identified a nonsense mutation (R156X) and a missense mutation (G116R) (Naz *et al.,* 2011). Another very recent study was carried out in 2011 by Naeem *et al.,* and identified a missense mutation p.D129G *GNAT1* in a large multigeneration family through the help of bi-directional sequencing. It normally is responsible for the adCSNB but in this family became a reason for autosomal recessive CSNB (Naeem *et al.,* 2011).

The objective of this current study is to collect and assemble more data about the symptoms and to seek for unknown genetic causes of inherited night blindness in Pakistani families. The families will be analyzed by using homozygosity mapping. This data will help in the generation of consensus based checklists which will lead to a more accurate and consistent diagnosis for benefit of humanity.

MATERIALS AND METHODS

In order to carry out the task of linkage studies of families with inherited night blindness, initially two families (A and B) with multiple affected individuals were identified and recruited from remote villages of Sindh province of Pakistan. At the time of visit all relevant information regarding family history including consanguinity and disease prevalence was collected. The data was saved in the form of pedigree constructed as described by Bennett *et al.,* (1995) using the software Cyrillic 2.1. In both pedigrees male and female members are symbolically represented by square and circles respectively. Normal members are represented by unfilled symbols while affected members are assigned filled symbols. Generation is conferred with Roman numeral and individuals in each generation are assigned Arabic numerals. Cousin marriages are indicated by the horizontal double lines between the respective couples.

2.1 Blood Sampling:

The blood Samples from normal and affected individuals of both families were collected with the consent of relevant persons using sterile 10 mL syringes and shifted into standard potassium ethylene diamine tetra acetate (EDTA) vacutainer tubes instantaneously (BD Vacutainer K3 EDTA, Franklin Lakes USA). Blood samples were then brought to laboratory in Department of Biochemistry, Quaid-i-Azam University, Islamabad and stored at 4 ^{0}C.

2.2 Clinical diagnostics:

In order to obtain details of clinical analysis of individuals suffering from disease, one individual from each family was taken to the ophthalmologist for examination to document clinical features. Ophthalmologic examination comprised of assessment of best-corrected visual acuity, external examination of eyes and slit lamp fundoscopy.

2.3 Extraction of Genomic DNA from Human Blood

For the extraction of whole genomic DNA two methods were employed depending upon the volume of the blood.

- Method using Organic Chemicals
- Method using Commercially available Kits

2.3.1 Organic Procedure of Extraction

Procedure started by taking 750 µL of blood in a microcentrifuge tube of 1.5 mL and solution A in an equal volume was put to it and for 5-10 minutes was incubated at room temperature. Centrifugation of these tubes was done in a microcentrifuge at 13,000 rpm (Eppendorf, Model 5417,Germany). Supernatant was thrown away cautiously subsequent to centrifugation and resuspension of pellet was done in 400 µL of solution A. For 1 minute, it was again centrifuged at 13,000 rpm. Supernatant obtained was again disposed of and pellet was dispersed in 400 µL of solution B, 12 µL of 20 % SDS and 20 µL of proteinase K (20 mg/mL) resuspension was done, and was left in incubator B28 (Binder, Germany) at 37 ^{O}C overnight. Following day freshly prepared mixture of equal volume 500 µL of solution C and D was added in eppendorf tubes, which were mixed and centrifuged for 10 mins at 13,000 rpm. The aqueous phase (upper layer) obtained was collected in a new 1/5 ml tube and was centrifuged at 13,000rpm for 10 minutes after addition of equal volume of solution D. After transferring the aqueous phase to a new 1.5ml tube for second time, 3M sodium acetate of pH 6 (55 µL) was put to it for precipitation of DNA and equal volume of isopropanol i.e 500 µL. Precipitation of DNA was done by inverting the tubes numerous times and centrifuged at 13,000 rpm for 10 minutes to settle down DNA. Finally Supernatant was thrown away and 200 µL of chilled 70 % ethanol was added for washing of pallet. For 8-10 minutes pellet was dried in incubator B28 afterwards (Binder, Germany) and disbanded in suitable quantity of Tris-EDTA (T.E) buffer that is 150-200 µL.

2.3.2 Constitution of Solutions

Solution A

$MgCl_2$	5 mM
Triton X-100	1%(v/v)
Tris pH 7.5	10mM
Sucrose	0.32 M

Solution B	EDTA pH 8	2mM
	Tris pH 7.5	10mM
	NaCl	400 mM
Solution C	Phenol	
Solution D	Chloroform	24 volumes
	Isoamyl alcohol	1 volume

Buffer for Dissolution of DNA(T.E)

Tris pH 8	10 mM
EDTA	0.1 mM

2.3.2 Extraction of DNA by using Commercial Kit:

Some of the DNA samples were also extracted by using Prime Prep ™Genomic DNA isolation Kit (Cat. No. K-2000, Genet Bio, Chungnam, Korea).In this method 20 uL of proteinase K solution was added to 1.5 mL microcentrifuge tube containing 200 mL of blood sample. Then 200 uL GB buffer was added to the sample and 15 minutes pulse vortexing was used for mixing it and kept it 56 °C for 10 minutes. In the next step 200 uL of absolute ethanol was added and mixed well by pulse vortexing for 15 seconds. Lysate was shifted into the upper reservoir of the spin column followed by centrifugation for 1 minute at 8000 rpm. After centrifugation the flow through was casted off and left over was shifted to a new 2ml collection tube. Later on 500ul GW2 solution was added to it and centrifuged again at 8000 rpm for 1minute and resulting flow through was discarded and centrifuged it once more at 12,000 rpm for 1-2 minutes for removal of ethanol. For elution the spin column was transferred to a new 1.5 mL tube and 200 uL GE buffer was added and kept for 1 minute at room temperature before centrifugation at 8000 rpm. Finally obtained genomic DNA was stored in Tris EDTA (TE) buffer.

2.4 Polymerase Chain Reaction (PCR)

With the help of site specific markers genomic DNA was amplified by using Polymerase chain reaction (PCR) given in Table 2.1. 25 µL PCR mixtures were prepared in 0.2 mL tubes (Axygen, USA) for each marker, with components mentioned below.

- 1 µL DNA (40 ng)
- 1.5 µL $MgCl_2$ (25 mM)
- 2.5 µL 10 X buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl)
- 0.5 µL dNTPs (10 mM, Fermentas, UK)
- 0.2 µL Taq DNA polymerase (5 U/µl, Fermentas, UK)
- 0.3 µL of each forward and reverse primer (0.1 µM)
- 18.7 µL PCR water.

All of the above stated ingredients were given a short spin for thorough mixing in centrifuge and amplification of this reaction mixture was carried out in thermocycler. Set Conditions for proper amplification are stated underneath. Preheating for the denaturation of the whole genome at 95 °C for 5 mins, 40 cycles of amplification immediately followed it, every cycle consisted of 3 steps: denaturation for 1 min at 95 °C, primer annealing at variable temperatures ranging between 50-57 °C and extension of DNA at 72 °C. Last step consists of 10 mins at 72 °C for the final extension of the amplified product. T1 thermocycler (Biometra, Germany) was used for PCR.

2.5 Horizontal Gel Electropherosis

The 2% agarose gel was used for observing resolution of PCR product of every marker. Protocol for preparation that was 0.8 g of agarose in 30mL of 10XTBE (0.89 M Tris-Borate, 0.032 M EDTA, pH 8.3) was dissolved which was heated in microwave oven and after the addition of 5 µL of ethidium bromide (0.5 µg/ml) for visualization of gel homogenized mixture was poured into gel cassette. Solidification of gel took 30 mins and PCR products with loading dye (0.25 % bromophenol blue with 40 % sucrose) were loaded into wells and ran at 110 volts (80mA) in 1XTBE for half an hour.

2.6 Vertical Gel Electrophoresis

In order to carry out vertical gel electrophoresis' the genotyping of every marker for each member of both families was carried out on 8% polyacrylamide, which was non-denaturing. It was prepared in a 250 ml conical flask and poured between two glass plates separated from each other at distance of 1.5mm by the help of spacers and comb. For polymerization it was kept at room temperature for approximately half an hour. After this glass plates were fixed with vertical gel tanks V16-2 (Life Technologies, USA) in a way of smaller plate facing inside the tank. Loading dye mixed with PCR products were loaded into wells. For almost two hours electrophoresis was performed. After the detachment of plates, gels were dipped into ethidium bromide with caution for staining. UV transiluminators in Gel Doc System (SYNGENE, UK) were employed for visualization of stained gels. Allele patterns obtained from each marker was scored by means of analyzing gels in both families.

2.6.1 Composition of 8 % Polyacrylamide Gel (50 mL)

- TEMED (N, N, N', N'-Tetra Methyl Ethylene Diamine) 17.5 µL
- 30 % Acrylamide solution (29 g polyacrylamide, 1 g NN'-Methylene-bisacrylamide) 13.5 mL
- 10 % Ammonium persulphate (APS) 350 µL
- 10 X TBE 5 ml
- distilled water 31.13 mL

2.7 Analysis of families with autosomal recessive night blindness:

In this study two families A and B with autosomal recessive congenital stationary night blindness were analyzed. Already identified loci/ genes for autosomal recessive night blindness were searched from National Center for Biotechnology Information (NCBI) Pubmed databases (http://www.ncbi.nlm.nih.gov/pubmed). The microsatellite markers (STS) flanking the genes identified through these searches (Table 2.1) were selected from University of California Santa Cruz (UCSC) genome browser and purchased from Gene Link (USA). For each candidate gene, a minimum of two STS markers were selected which flanked the respective candidate gene. After amplification of each marker specified in table 2.1, PCR products were resolved on 8% non-denaturing polyacrylamide gel as described above. Gel was stained with

ethidium bromide and UV transilluminator in Gel Doc system (SYNGENE, UK) was used for visualization. Allelic pattern in normal and affected members of each family was analyzed by the images obtained for each marker. To find disease associated homozygosity by descent (HBD) in affected individuals of each family the data was later analyzed. For the calculation possibility of linkage and exclusion of candidate genes in tested families the data was also analyzed with easy LINKAGE plus V 5.02. The disease allele frequency of 0.001, equal marker allele frequency and recessive mode of inheritance were the parameters used for linkage analysis.

Table 2.1: Catalog of microsatellite markers for analysis of families with autosomal recessive CSNB

S. No	Genes /loci	Chromosomal Location	Markers	Map Distance (cM) *
1.	*SAG*	2q37.1	D2S1363 D2S427 D2S2344 D2S2176 D2S331	231.1 237 242.83 243.8 244.45
2.	*CCDC66*	3p14.3	D3S2400 D3S2400 D3S3616 D3S2402	75.23 75.08 76.16 76.71
3.	*GRM6*	5q35.3	D5S1398 D5S2863 D5S2008 D5S2073 D5S135	199.02 201.59 202.74 206.62 207.73
4.	*CABP4*	11q13.2	D11S1368 D11S913 D11S987	70.24 76.62 77.28
5.	*RDH5*	12q13.2	D12S2206 D12S376 D12S326	89 89.61 93
6.	*GRK1*	13q34	D13S1265 D13S743 D13S1295 D13S293	115.52 116.59 127.43 130.41
7.	*TRPM1*	15q13.3	D15S217 D15S1043 D15S165 D15S976 D15S231	14.46 19.89 20.5 21.01 24.67
8.	*SLC24A1*	15q22.31	D15S1507 D15153 D15S988	64.41 66.22 68.64

RESULTS

3.1 Families Studied:

3.1.1 Family A:

Family A show signs of autosomal recessive congenital stationary night blindness. Pedigree of Family A (Figure 3.1) has four generations consisting of 14 individuals. Three individuals (IV-4, IV-5, IV-6) show phenotypic expression of night blindness while the rest of two (III-2, IV-2) were normal. As the parents show no symptoms of night blindness this suggested autosomal mode of inheritance. Blood samples of normal (III-2, IV-2) and affected (IV-4, IV-5, IV-6) individuals were drawn for genomic DNA isolation and subsequent linkage analysis.

3.1.2 Family B:

An autosomal recessive mode of inheritance of night blindness is exhibited in the pedigree of Family B which has six generations comprising of 29 individuals. Six individuals (V-7, V-8, V-10, VI-2, VI-3, VI-4) show phenotypic expression of night blindness and rest of individuals (V-6, V-9, VI-1, VI-5). Blood samples of normal (V-6, V-9, VI-1, VI-5) and affected (V-7, V-8, V-10, VI-2, VI-3, VI-4) individuals were collected for linkage analysis.

3.1.3 Clinical Presentations of Family A and B:

Clinical presentations of affected individuals revealed the presence of congenital stationary night blindness. General eye examination, visual acuity, fundoscopic examination and ERG findings all support the presence of congential stationary night blindness. General examination and interviews regarding the complete medical history of the individuals and family relationships revealed that there was no possibility of blindness due to environmental factors and infections. They were physically and mentally normal except ocular disorder. Patients also reported consistent difficulties in seeing at night, even when they were fully dark-adapted. They could not detect objects readily visible to others and showed both confusion and slow recovery after brief exposure to relatively bright light sources. The visual acuity of the patients was also low. The affected members of family B had the symptoms of night blindness such as loss of vision, near sightedness and non progressive night blindness. The vision at day time was normal and affected persons showed blindness at night or at low light levels. A member in the pedigree had vision lost due to some

nervous problem. The disease was stationary as it was non progressive and conditions of the patients did not get worse with the age.

3.2 Linkage Analysis of Families A and B:

On the basis of clinical analysis congenital stationary night blindnesss was suggested for both families. In order to idenitify the genetic defects of the disease linkage analysis was done in both families. This was done by using STS markers for already known genes involved in autosomal recessive night blindness. Genes which are present on the same chromosome are said to be linked and the tendecncy of parental recombinations to remain together is called linkage (Gardner *et al.,* 1991). As the family B shows an X-linked mode of inheritance so direct sequencing of a gene was carried out to identify pathogenic mutation.

3.2.1 Family A:

DNA samples of four individuals including two normal (III-2, IV-2) and two affected (IV-5, IV-6) having phenotypic expression of congenital stationary night blindness were genotyped by using microsatellite markers listed in table 2.1. Analysis of the results was done to score in order to the homozygosity pattern which is shared among all diseased members of the family.From the analysis of genotyped results it was clear that all affected individuals show heterozygosity for parental alleles for all tested loci (Fig 3.3-3.22). Moreover genotyped data was also analyzed using easy LINKAGE plus V 5.02 (Linder & Hoffmann, 2005) which yielded maximum two point LOD score of 0.20 at marker D13S1265 flanking the gene *GRK1* (Fig 3.23). Absence of homozygosity in all affected individuals and no significant LOD score exclude (Table 2.1) linkage in family A to known genes/loci.

3.2.2 Family B:

DNA samples of members of the family including four normal (V-6, V-9, VI-1, VI-5) and six affected (V-7, V-8, V-10, VI-2, VI-3, VI-4) individuals having phenotypic expression of congenital stationary night blindness were genotyped using microsatellite markers listed in table 2.1. Results of the genotyping were analyzed to score alleles to find homozygosity pattern shared by all affected individuals (Fig 3.24-3.40). Analysis of results obtained from the genotyped markers made it clear that all diseased individuals show heterozygosity to parental alleles for all tested loci. The whole genotyped data was analyzed using easy LINKAGE plus V 5.02 which yielded

maximum two point LOD score of 1.46 at marker D13S1295 flanking the gene *GRK1* (Fig 3.41). These results showed an absence of homozygosity in all the affected individuals for tested genes/loci.

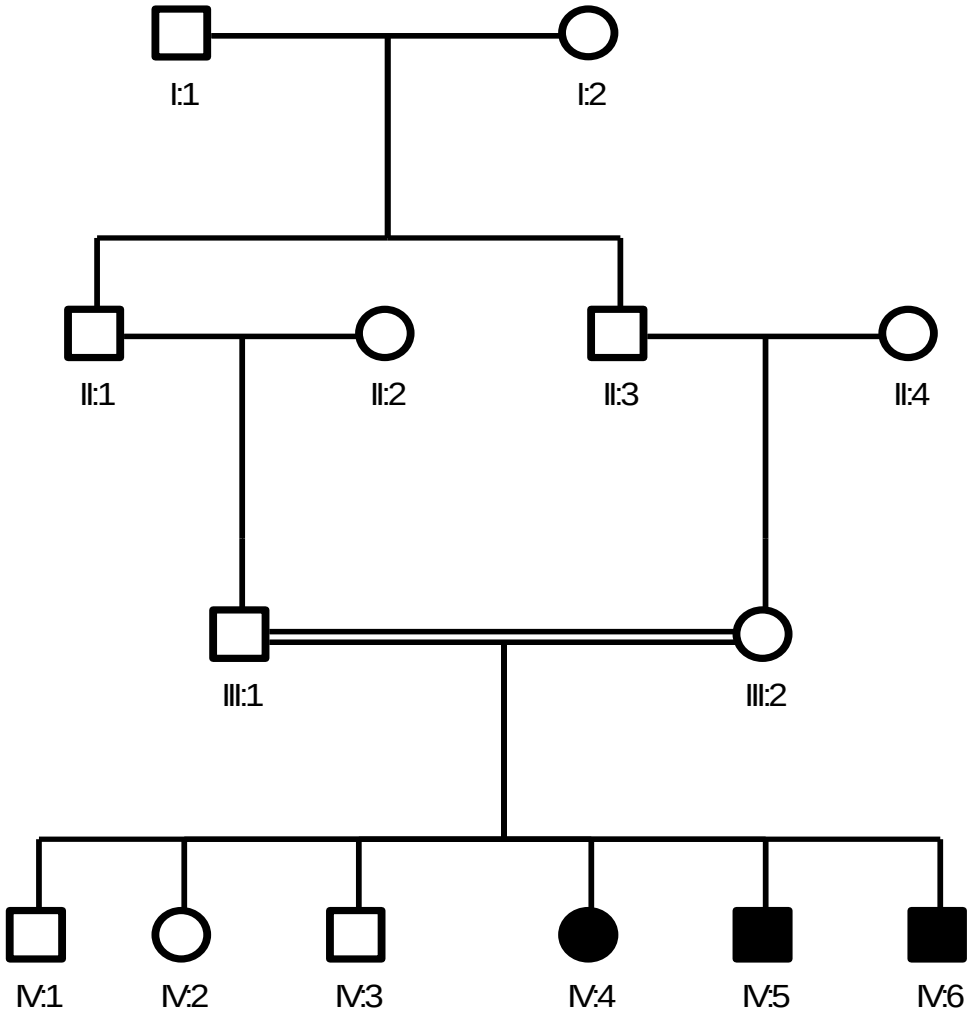

Figure 3.1: Pattern of autosomal recessive inheritance is exhibited by Pedigree of family A with night blindness. Females are represented by circles while males by squares. Disaesed individuals are indicated by filled icons while normal individuals by unfilled. Consanguineous marriages are displayed by double lines.

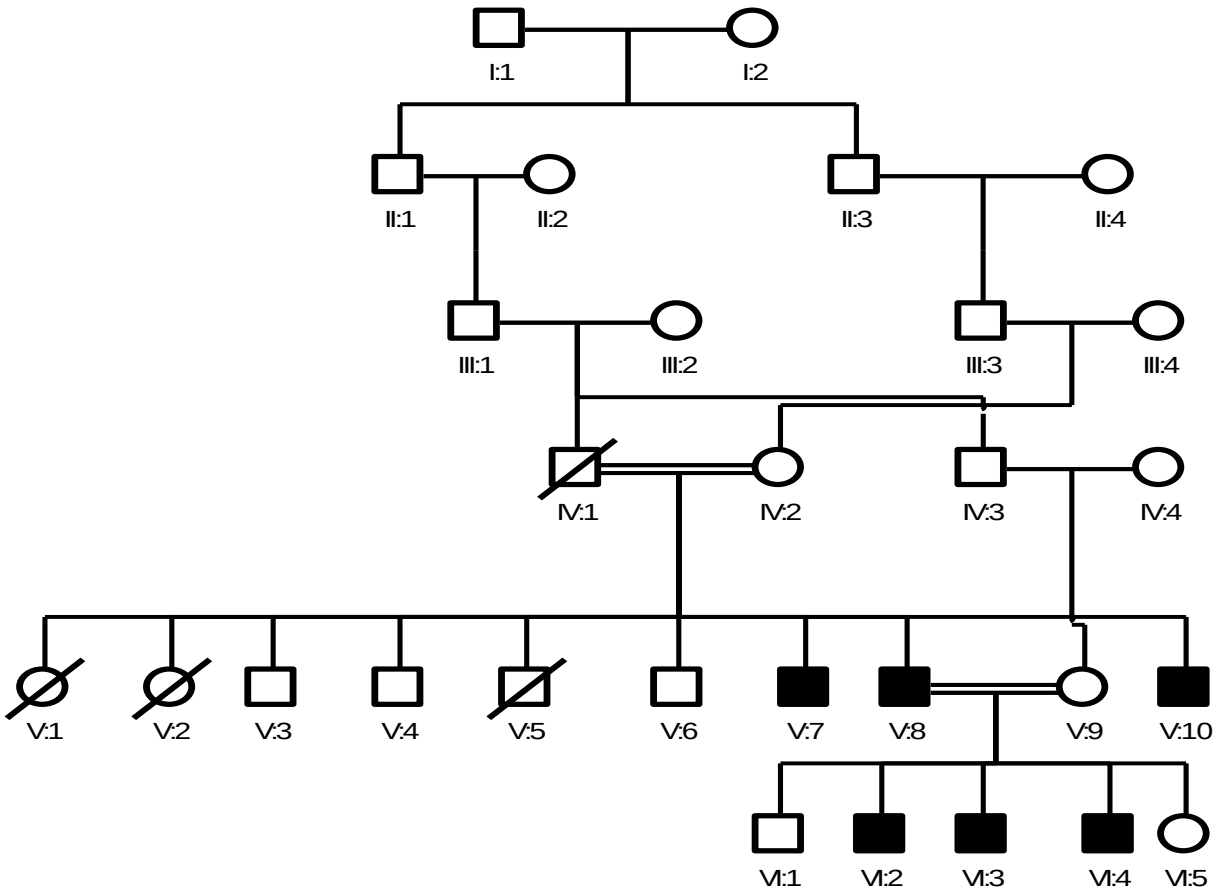

Figure 3.2: Pattern of autosomal recessive inheritance is exhibited by Pedigree of family B with night blindness. Females are represented by circles while males by squares. Diseased individuals are indicated by filled icons while normal individuals by unfilled. Consanguineous marriages are displayed by double lines.

Individual ID	IV-6	IV-5	IV-2	III-2
Status	D	D	N	N

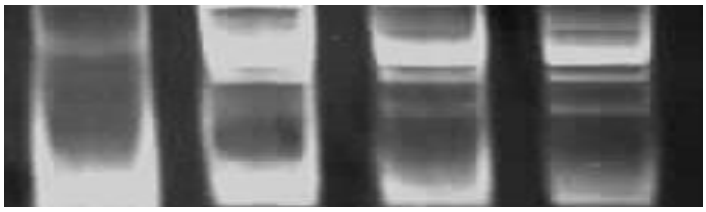

Figure 3.3: Allelic pattern obtained with marker D2S2176 at 243.8 cM in family A. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one.

Individual ID	IV-6	IV-5	IV-2	III-2
Status	D	D	N	N

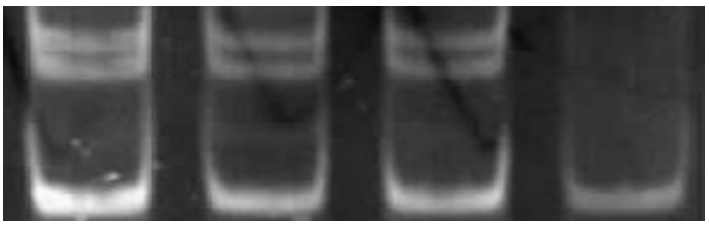

Figure 3.4: Allelic pattern obtained with marker D2S2968 at 244.2 cM in family A. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one.

Individual ID	IV-6	IV-5	IV-2	III-2
Status	D	D	N	N

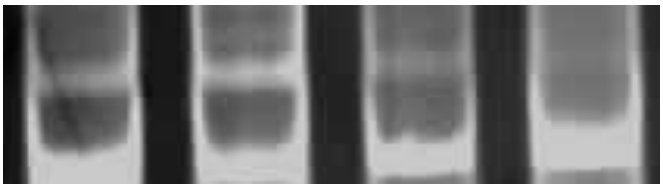

Figure 3.5: Allelic pattern obtained with marker D3S2402 at 76.71 cM in family A. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one

Individual ID	IV-6	IV-5	IV-2	III-2
Status	D	D	N	N

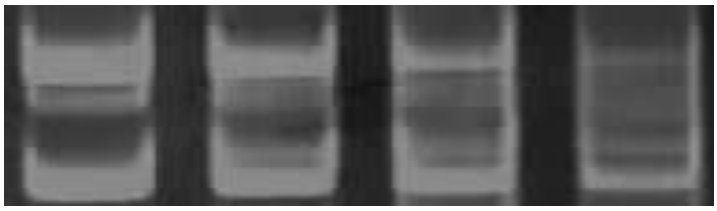

Figure 3.6: Allelic pattern obtained with marker D3S2400 at 75.23 cM in family A. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one

Individual ID	IV-6	IV-5	IV-2	III-2
Status	D	D	N	N

Figure 3.7: Allelic pattern obtained with marker D5S2863 at 201.59 cM in family A. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one

Individual ID	IV-6	IV-5	IV-2	III-2
Status	D	D	N	N

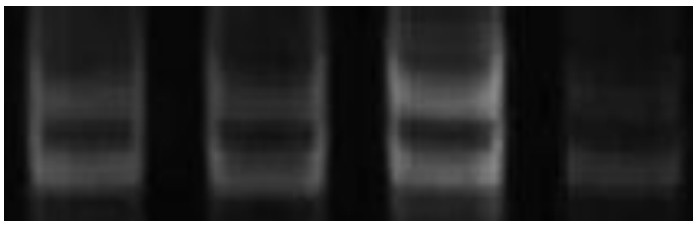

Figure 3.8: Allelic pattern obtained with marker D5S2006 at 202.74cM in family A. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one

Individual ID	IV-6	IV-5	IV-2	III-2
Status	D	D	N	N

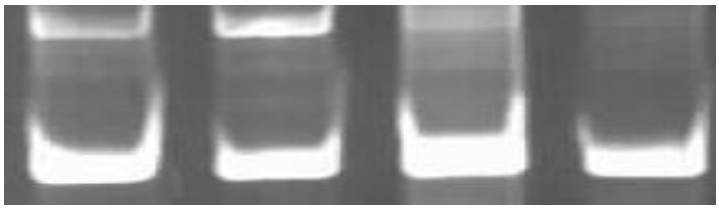

Figure 3.9: Allelic pattern obtained with marker D5S153 at 207.73 cM in family A. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one

Individual ID	IV-6	IV-5	IV-2	III-2
Status	D	D	N	N

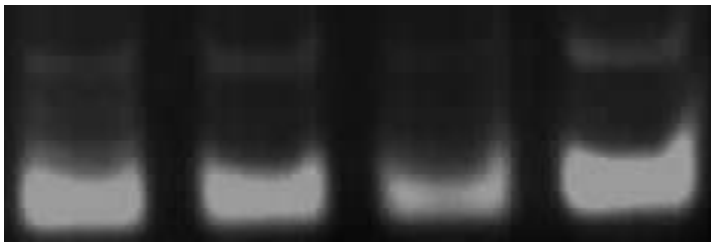

Figure 3.10: Allelic pattern obtained with marker D11S913 at 76.62 cM in family A. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one

Individual ID	IV-6	IV-5	IV-2	III-2
Status	D	D	N	N

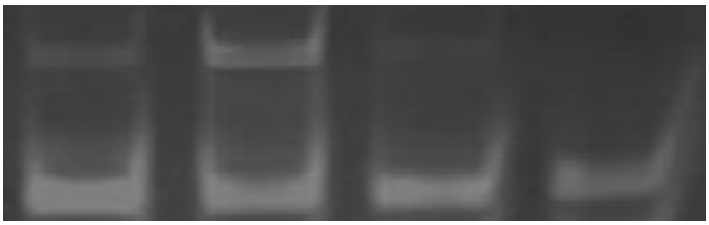

Figure 3.11: Allelic pattern obtained with marker D11S1368 at 70.24 cM in family A. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one

Individual ID	IV-6	IV-5	IV-2	III-2
Status	D	D	N	N

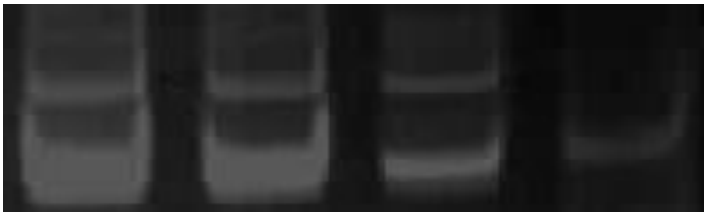

Figure 3.12: Allelic pattern obtained with marker D12S376 at 89.61 cM in family A. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one

Individual ID	IV-6	IV-5	IV-2	III-2
Status	D	D	N	N

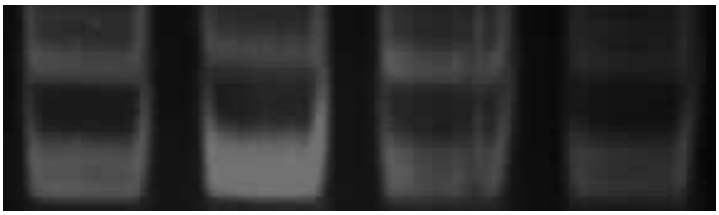

Figure 3.13: Allelic pattern obtained with marker D13S293 at 130.42 cM in family A. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one

Individual ID	IV-6	IV-5	IV-2	III-2
Status	D	D	N	N

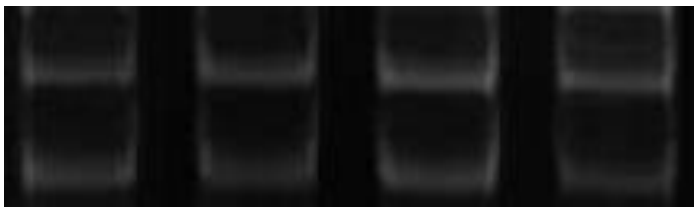

Figure 3.14: Allelic pattern obtained with marker D15S1043 at 19.89 cM in family A. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one

Individual ID	IV-6	IV-5	IV-2	III-2
Status	D	D	N	N

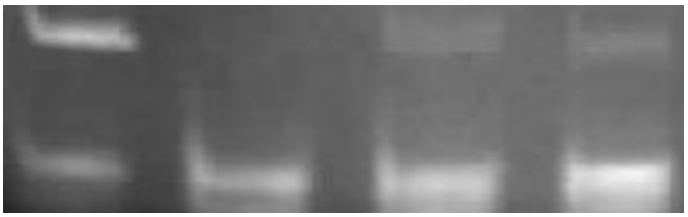

Figure 3.15: Allelic pattern obtained with marker D15S231 at 24.67 cM in family A. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one.

Individual ID	IV-6	IV-5	IV-2	III-2
Status	D	D	N	N

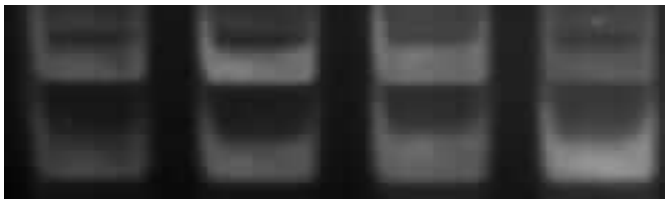

Figure 3.16: Allelic pattern obtained with marker D15S153 at 66.22 cM in family A. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one

Individual ID	IV-6	IV-5	IV-2	III-2
Status	D	D	N	N

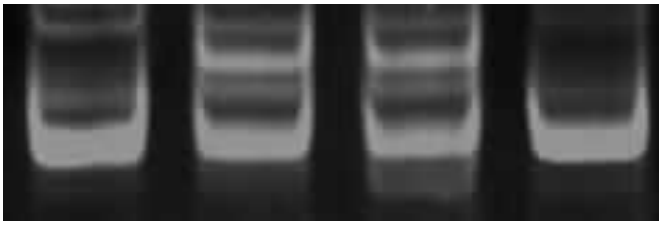

Figure 3.17: Allelic pattern obtained with marker D15S988 at 68.73 cM in family A. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one

Project:	MA98	Inheritance:	Recessive
Family name:	TOTALS	Common allele:	99.90 %
Used map:	Marshfield (sex averaged)	Disease allele:	0.10 %
Marker positions:	19 ok / 2 ? / 0 outside	LC1 PCOPY rate:	0.00 %
Allele frequencies:	All individuals (marker file)	LC1 PENET wt/mt:	0.00 %
CALC interval:	Entire chromosome	LC1 PENET mt/mt:	100.00 %

Marker	CHR	cM	LOD	Theta
1.D13S1265	13	98.82	0.2088	0.0000
2.D3S2402	3	77.01	0.0073	0.2500
3.D5S1354	5	194.88	0.0073	0.2500
4.D11S1368	11	59.78	0.0073	0.2500
5.D2S2176	2	242.17	0.0000	0.5000

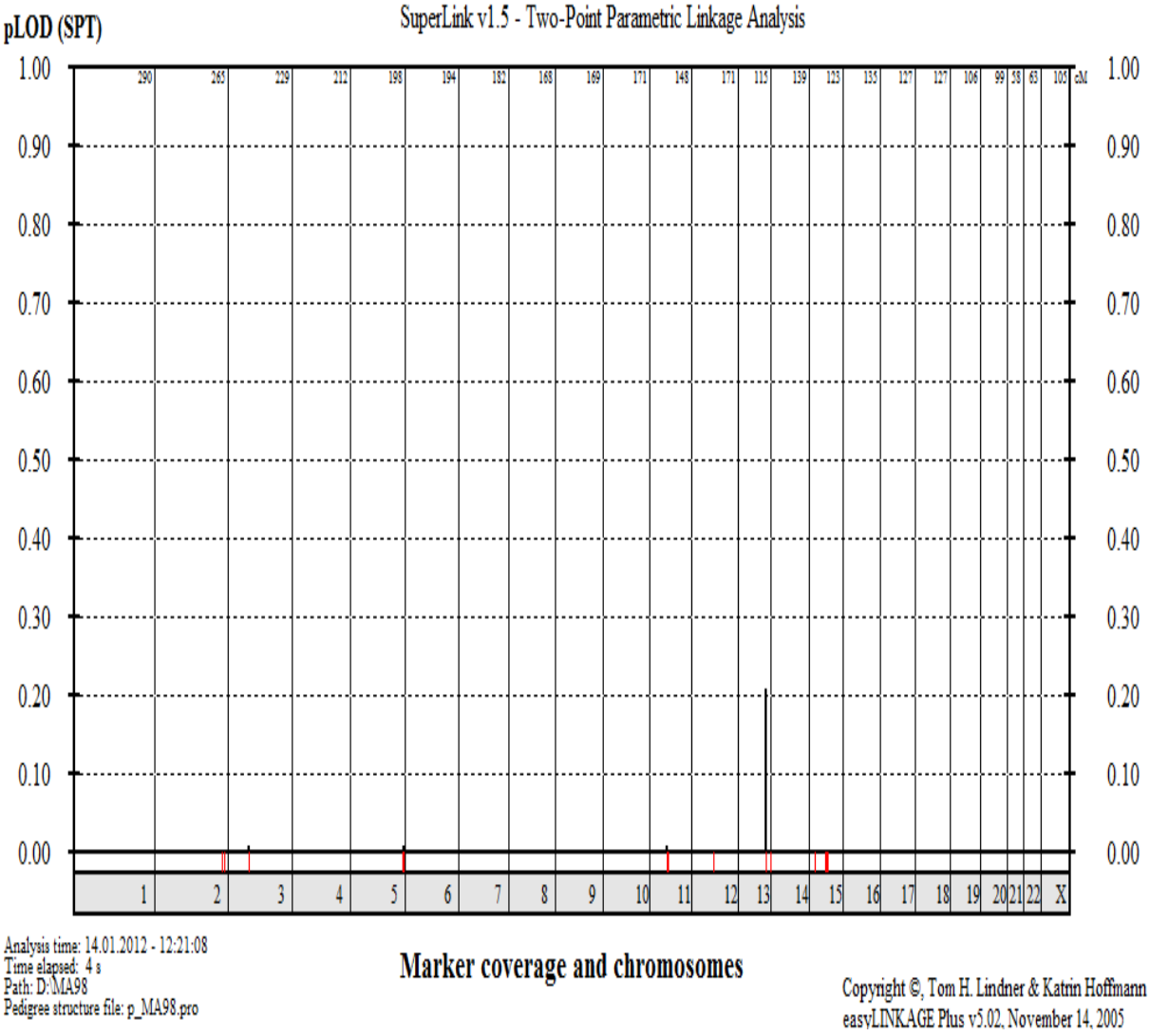

Figure 3.18: Depiction of the LOD score in the form of a Graph attained for markers genotyped in Family A.The marker D13S1265 yeilded maximum LOD score of 0.2008 by using easyLINKAGE V 5.02.

Individual ID

	V-10	V-7	VI-4	VI-1	VI-2	VI-5	VI-3	V-9	V-8	V-6
Status	D	D	D	N	D	N	D	N	D	N

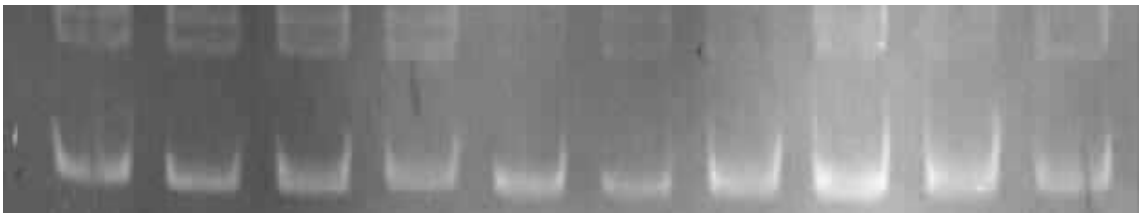

Figure 3.19: Allelic pattern obtained with marker D2S2968 at 243.34 cM in family B. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one.

Individual ID

	V-10	V-7	VI-4	VI-1	VI-2	VI-5	VI-3	V-9	V-8	V-6
Status	D	D	D	N	D	N	D	N	D	N

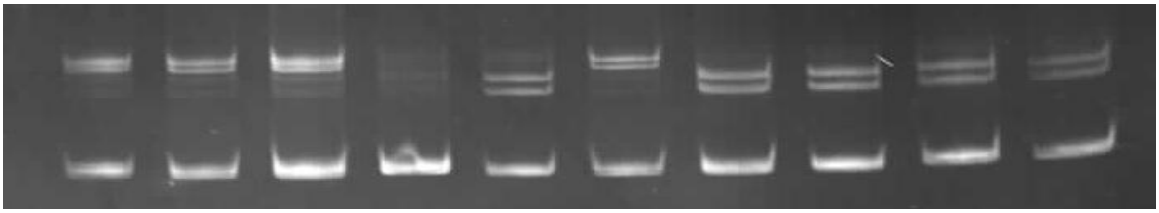

Figure 3.20: Allelic pattern obtained with marker D2S427 at 237 cM in family B. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one.

Individual ID

	V-10	V-7	VI-4	VI-1	VI-2	VI-5	VI-3	V-9	V-8	V-6
Status	D	D	D	N	D	N	D	N	D	N

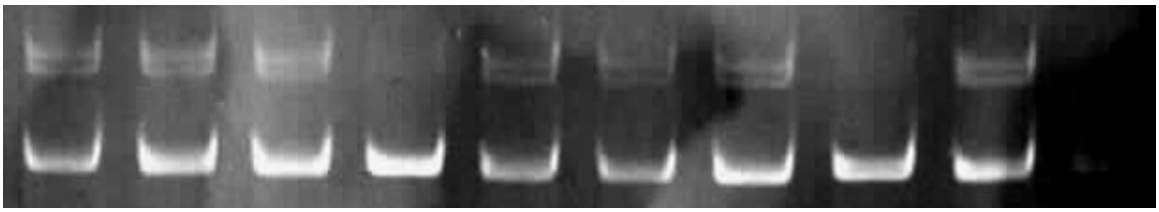

Figure 3.21: Allelic pattern obtained with marker D3S3616 at 76.16 cM in family B. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one.

Individual ID

	V-10	V-7	VI-4	VI-1	VI-2	VI-5	VI-3	V-9	V-8	V-6
Status	D	D	D	N	D	N	D	N	D	N

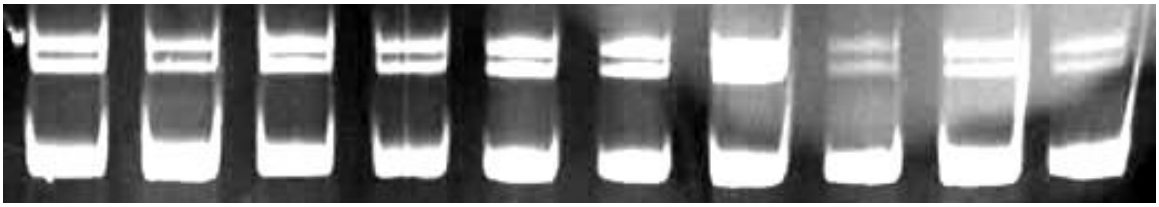

Figure 3.22: Allelic pattern obtained with marker D3S3048 at 75.08 cM in family B. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one.

Individual ID

	V-10	V-7	VI-4	VI-1	VI-2	VI-5	VI-3	V-9	V-8	V-6
Status	D	D	D	N	D	N	D	N	D	N

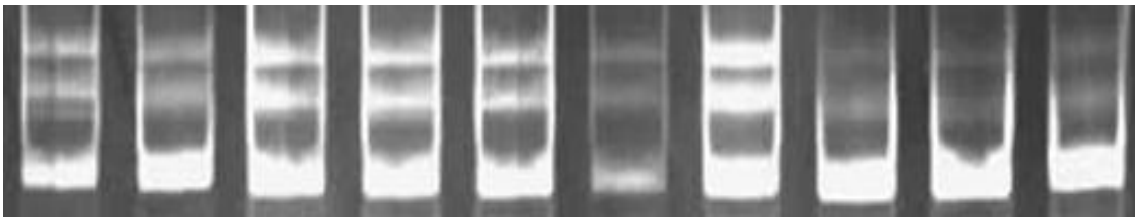

Figure 3.23: Allelic pattern obtained with marker D3S2402 at 76.71 cM in family B. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one.

Individual ID

	V-10	V-7	VI-4	VI-1	VI-2	VI-5	VI-3	V-9	V-8	V-6
Status	D	D	D	N	D	N	D	N	D	N

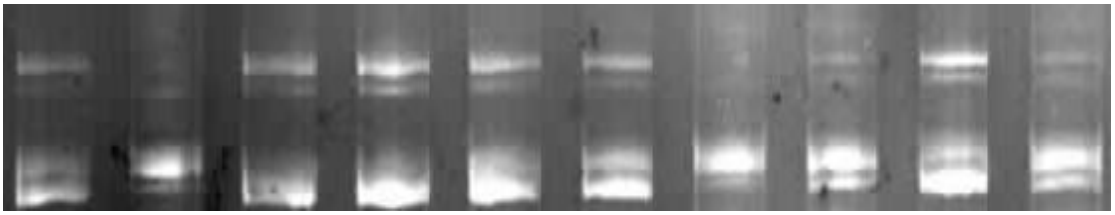

Figure 3.24: Allelic pattern obtained with marker D5S2073 at 206.6 cM in family B. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one.

Individual ID

	V-10	V-7	VI-4	VI-1	VI-2	VI-5	VI-3	V-9	V-8	V-6
Status	D	D	D	N	D	N	D	N	D	N

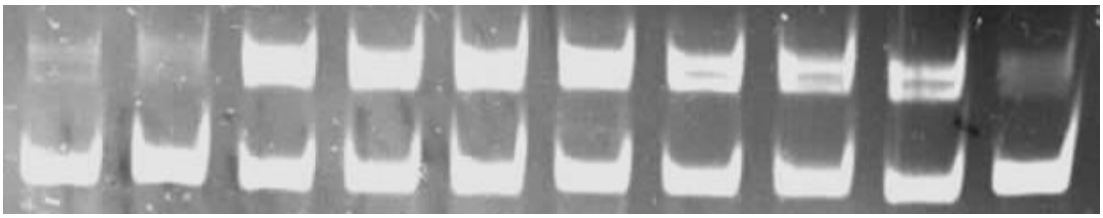

Figure 3.25: Allelic pattern obtained with marker D5S1354 at 207.23 cM in family B. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one.

Individual ID

	V-10	V-7	VI-4	VI-1	VI-2	VI-5	VI-3	V-9	V-8	V-6
Status	D	D	D	N	D	N	D	N	D	N

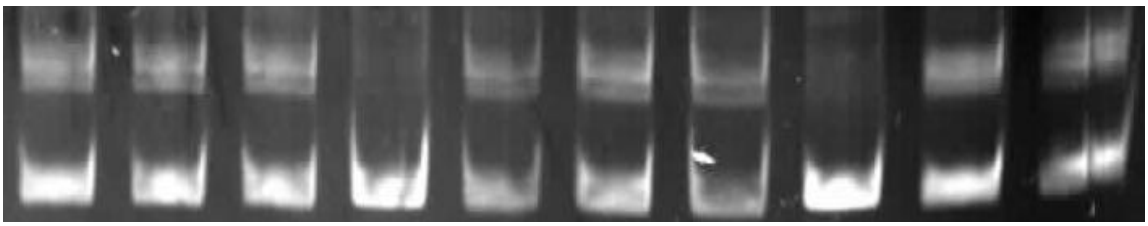

Figure 3.26: Allelic pattern obtained with marker D11S1368 at 70.24 cM in family B. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one.

Individual ID

	V-10	V-7	VI-4	VI-1	VI-2	VI-5	VI-3	V-9	V-8	V-6
Status	D	D	D	N	D	N	D	N	D	N

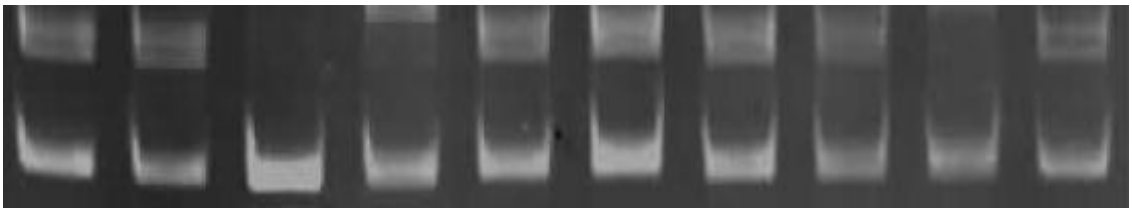

Figure 3.27: Allelic pattern obtained with marker D12S376 at 89 cM in family B. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one.

Individual ID

	V-10	V-7	VI-4	VI-1	VI-2	VI-5	VI-3	V-9	V-8	V-6
Status	D	D	D	N	D	N	D	N	D	N

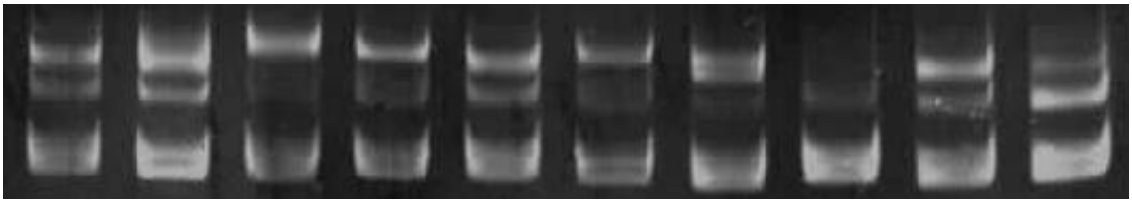

Figure 3.28: Allelic pattern obtained with marker D12S326 at 93 cM in family B. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one.

Individual ID

	V-10	V-7	VI-4	VI-1	VI-2	VI-5	VI-3	V-9	V-8	V-6
Status	D	D	D	N	D	N	D	N	D	N

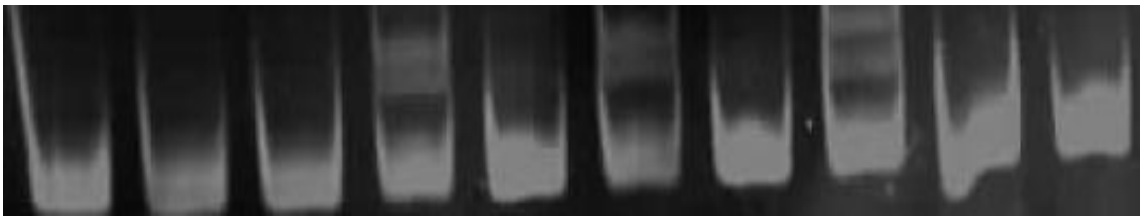

Figure 3.29: Allelic pattern obtained with marker D13S1295 at 110.55 cM in family B. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one.

Individual ID

	V-10	V-7	VI-4	VI-1	VI-2	VI-5	VI-3	V-9	V-8	V-6
Status	D	D	D	N	D	N	D	N	D	N

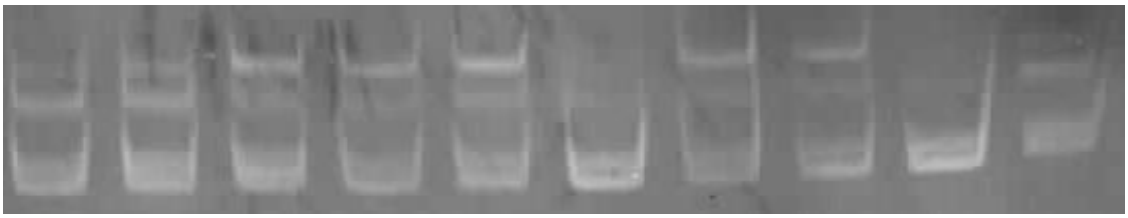

Figure 3.30: Allelic pattern obtained with marker D13S1315 at 117.59 cM in family B. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one.

Individual ID

	V-10	V-7	VI-4	VI-1	VI-2	VI-5	VI-3	V-9	V-8	V-6
Status	D	D	D	N	D	N	D	N	D	N

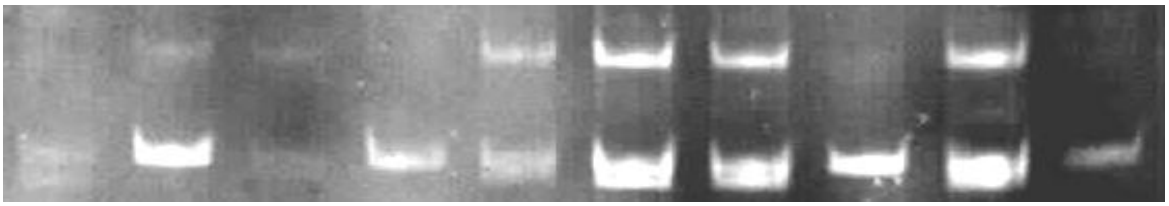

Figure 3.31: Allelic pattern obtained with marker D15S217 at 14.46 cM in family B. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one.

Individual ID

	V-10	V-7	VI-4	VI-1	VI-2	VI-5	VI-3	V-9	V-8	V-6
Status	D	D	D	N	D	N	D	N	D	N

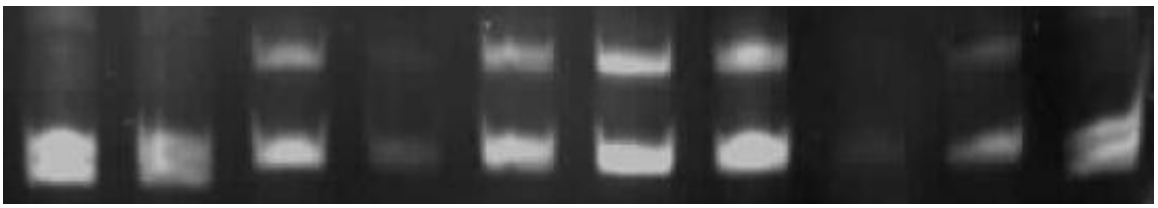

Figure 3.32: Allelic pattern obtained with marker D15S231 at 24.67 cM in family B. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one.

Individual ID

	V-10	V-7	VI-4	VI-1	VI-2	VI-5	VI-3	V-9	V-8	V-6
Status	D	D	D	N	D	N	D	N	D	N

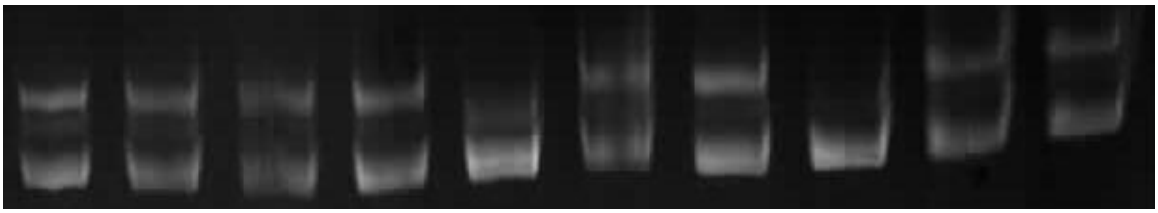

Figure 3.33: Allelic pattern obtained with marker D15S988 at 68.64 cM in family B. Numbering refers to position of individual in the pedigree. D corresponds to diseased individual while N to normal one.

Project:	MA100	Inheritance:	Recessive
Family name:	TOTALS	Common allele:	99.90 %
Used map:	Marshfield (sex averaged)	Disease allele:	0.10 %
Marker positions:	15 ok / 1 ? / 0 outside	LC1 PCOPY rate:	0.00 %
Allele frequencies:	All individuals (marker file)	LC1 PENET wt/mt:	0.00 %
CALC interval:	Entire chromosome	LC1 PENET mt/mt:	100.00 %

Marker	CHR	cM	LOD	Theta
1.D13S1295	13	110.55	1.4621	0.0000
2.D3S3616	3	76.48	0.1822	0.1500
3.D2S1363	2	227.00	0.0003	0.4500
4.D2S427	2	236.70	0.0000	0.0000
5.D3S3048	3	75.41	0.0000	0.5000

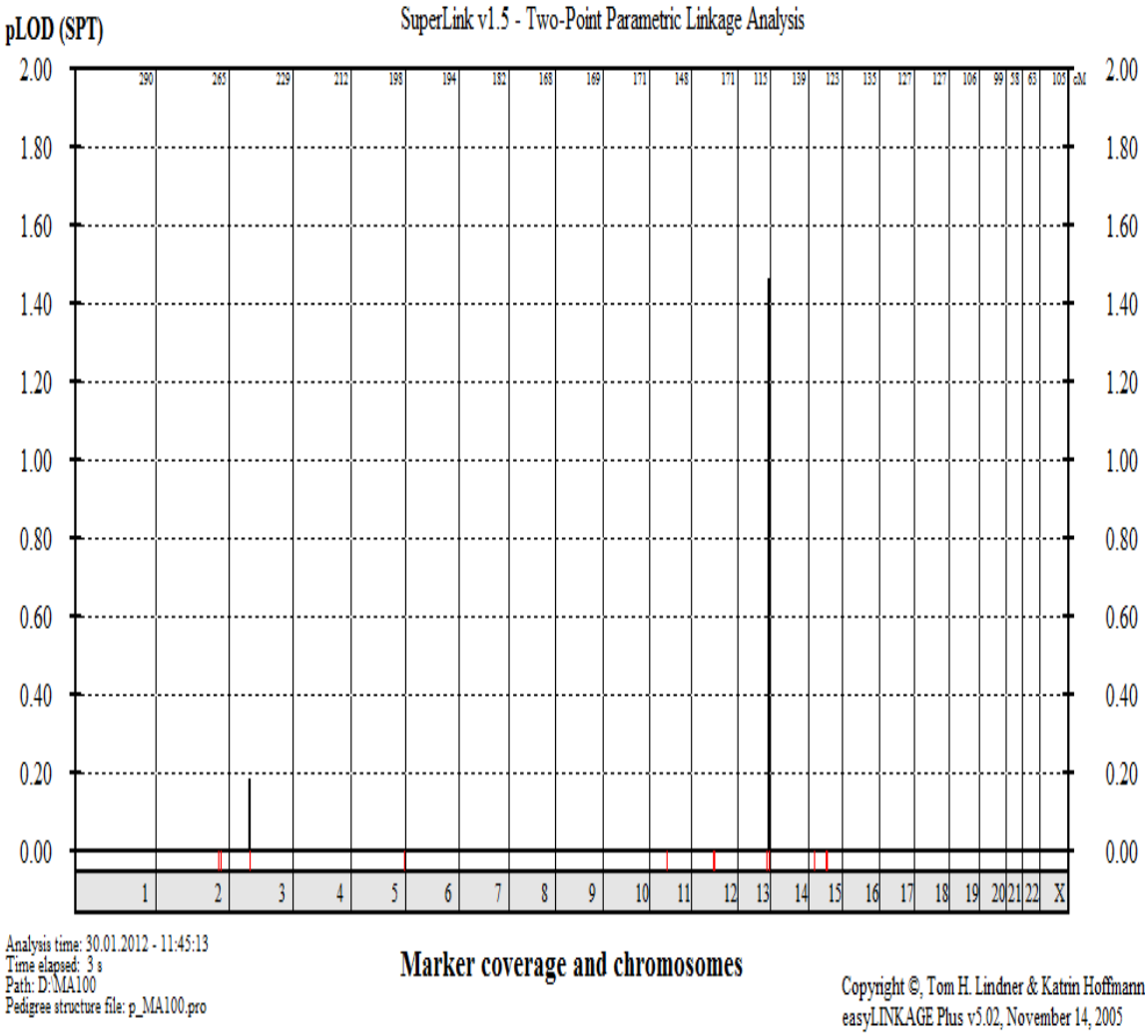

Figure 3.34: Depiction of the LOD score in the form of a Graph attained for markers genotyped in Family B. The marker D13S1295 yeilded the maximum LOD score of 1.46 by using easyLINKAGE V 5.02.

DISCUSSION

A hereditary retinal disease mostly resulting from the dysfunction in the retinal cells is congenital stationary night blindness (CSNB) (Aldamesh *et al.,* 2010). CSNB is a functional defect which makes it much different from other retinal dystrophies (Szabo *et al.,* 2007) as CSNB is caused by the defect in the process of signaling from photoreceptor to bipolar cells (Zeitz *et al*., 2007, 2009). In this disorder there is an enduring scarce vision in the dark, as rod cells gets desensitized in stunned vision under dim light conditions because of consistent little basal stimulation (Rebhun *et al.,* 1984; Sandmeyer *et al.,* 2007). Desensitization is because of constitutively active mutant opsins (Dryja *et al.,* 1993), or thermal isomerization of the chromophore which causes activation of mutant rhodopsin in absence of light (Sieving *et al.,* 1995) or triggering activation of rhodopsin.

The present study involves two families A and B having clinical presentations of congenital stationary night blindness, screened for the determination of genetic causes. These families exhibited a recessive mode of inheritance. In order to find the genetic basis of the disease, linkage analysis a family based approach was used for mapping of disease that ran across this pedigree. Within a family specific replicas of genomic region anchoring the disease genes are co-inherited which reflects a non existence of recombination between the disease mutation and neighboring genetic markers due to their close proximity. Individuals who share a disease within a family will typically contribute to alleles near the marker region (Ott, 1991). The classical technique employed for studying the linkage in consanguinity is Homozygosity Mapping that has been used for almost three decades as a powerful research methodology through which countless hereditary diseases have been mapped (Lander and Botstein, 1987; Seelow *et al.,* 2009; Singh *et al.,* 2009). The homozygosity-by-decent (HBD) or allelic identity-by-decent (IBD) is that homozygosity which occurs as a consequence of consanguineous marriges. Homozygous region of 6% (1/16) is shared amongst individuals born of consanguineous unions in their genome (Sheffield *et al.,* 1998) which is consistent to a size of 20 cM (Wright *et al.,* 1997).

Results obtained by linkage analysis are further detected by LOD score which is a statistical representation of the relative likelihood of a disease locus and genetic marker are genetically linked, rather than they are genetically unlinked (Ott, 1991;

Strachan 1992; Strachan and Reads, 1996). Several factors such as certainty of clinical diagnosis, the size of the family being examined, the number of affected individuals and their location within the pidegree, mode of inheritance, and informativeness of the genetic markers used influence the LOD score (Damji and Allingham, 1997).

Seven genes responsible for autosomal recessive CSNB *(SAG, CCDC66, GRM6, CABP4, RDH5*, *TRPM1* and *SLC24A1)* were checked for linkage analysis in this study. Linkage analysis was done on both families but none of the genes was found to be linked in both families. It was further confirmed through the calculation of LOD score. The highest LOD score of 0.20 was obtained for Family A which confirmed the exclusion of known genes. It was at marker D13S1265 flanking the gene *GRK1*. This value of LOD score may be because of the small size of family as only two affected individuals and two normal individuals were present. In the family B ten individuals were analyzed including six affected and four normal but no gene was found to be linked. A highest LOD score of 1.46 was obtained for this family at D13S1295. This marker flanks *GRK1* gene which is responsible for the cause of Oguchi disease, a variant of CSNB (Zhang *et al.*, 2005; Hayashi *et al.,* 2007). In both families the maximum LOD score was obtained at makers flanking for *GRK1* gene and mutations in this gene have already been reported in Japanese and European population (Yamamoto *et al.*, 1997; Hayashi *et al.*, 2007; Oishi *et al.*, 2007). *GRK1* is G-protein dependent receptor kinase 1, also called as Rhodopsin kinase is a rod specific cytosolic enzyme. It stops the phototransduction cascade by the deactivation of the photoactivated rhodopsin (Young *et al.*, 2007).

A Pakistani family manifesting clinical presentations like an atypical Mizuo-Nakamura phenomenon, a non-recordable rod ERG, cone response on ERG recording, caliber of retinal blood vessels and visual acuity, with all normal readings suggest retinal dysfunction rather than degeneration differing from typical form of Oguchi was reported by Zhang *et al.*, in (2005) having mutations in *GRK1* gene. A novel mutation in *GRK1* responsible for Oguchi disease have been reported in (2007) by Hayashi *et al.*, in two Japense patients, from a consanguineous family. The patients had night blindness, normal color vision, a typical fundus discoloration with golden-yellow appearance, no rod ERGs but markedly reduced single flash cone (Hayashi *et al.*, 2007). Azam *et al.*, (2009) provided testimonial of a novel mutation in *GRK1* in a

large Pakistani family having typical Oguchi disease. The affected members suffered from congenital stationary night blindness showing the Mizuo-Nakamura phenomenon after dark adaptation along with dispersed gray discoloration of the fundus. Rod responses were nonrecordable as shown by ERG data. The affected members of families in our study manifested symptoms of night blindness such as, low visual acuity, loss of vision, near sightedness and non progressive congenital night blindness. The vision at day time was normal and affected persons showed blindness at night or at low light levels. Some of the clinical features of the families reported for Oguchi disease are consistent with the presentations of families studied so there is a chance of occurrence of any change in this gene and it may be susceptible for the disease in our case. This can be further confirmed by the application of fine homozygosity mapping and more advance techniques.

As exclusion has been obtained in both of the cases, current research conducted suggests further study for more innovative techniques such as the whole genome scan including SNP based genetic linkage analysis for the identification and mapping of new disease loci and to determine genes responsible for night blindness in order to understand pathogenesis of the disease.

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- RetNet - Retinal Information Network:
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