Molecular Genetic Studies of Childhood Blindness

Vafa Keser

Department of Human Genetics

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Supervisor Robert K. Koenekoop MD PhD

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ABSTRACT

Childhood blindness due to hereditary retinal disease represents a huge clinical problem. Morbidity, blindness but also a major lack of understanding the molecular genetic events that lead to blindness in very young children hamper clinical management of this important and common problem. Blindness is a multifactorial disease with genetic, environmental, optical, developmental and local ocular components. One way to get entry into the disease mechanism of childhood retinal blindness is to study Mendelian disease entities that are caused by relatively straightforward links between mutations in genes that lead to aberrant retinal protein function and the resulting disease phenotype. These discoveries in the Mendelian model would lead to understanding of the genetics, proteomics and most importantly retinal disease mechanisms and cycles that underlie the more complex diseases causing blindness. This would lead to clinically relevant information to guide ophthalmologists, scientists and patients.

According to the World Health Organization (WHO) there are approximately 1.5 million blind children in the world and two-thirds of them are in Asia. Inherited blindness comprises 66% of childhood blindness, the largest subgroup. In the Middle East, in Pakistan and in other societies, blindness is relatively more common due to consanguineous marriages [1-4].

In collaboration with a group of geneticists, ophthalmologists and fellows in Islamabad, Pakistan where consanguineous marriages are also common, we have entered into a very fruitful international collaboration with whom we have assembled a very exciting collection of 21 consanguineous pedigrees with a

devastating autosomal recessive Mendelian disease, congenital retinal detachment, also known as nonsyndromic congenital retinal nonattachment (NCRNA) and 16 families with autosomal recessive retinitis pigmentosa (arRP). We collected the phenotypes, obtained the scientific and ethical approvals, signed the consents and collected the DNA on these families to conduct our genetic analysis.

Our main guiding hypothesis is that a significant portion of NCRNA will be caused by mutations in novel retinal genes as currently only one NCRNA gene is known (*ATOH7*). These novel genes may give us insight into the disease pathways of retinal detachment itself, a huge and clinically unmet medical need. Adult retinal detachments are common and the disease pathways are unknown.

In a preliminary study of our cohort, we performed SNP genotyping where we aimed to identify homozygous (hmz) regions. Homozygous regions contain the causal gene(s) in consanguineous families. We found that in many of our patients known retinal detachment or RP gene(s) resided in one of the top 5 largest homozygous segments documented by the SNP array. Direct sequencing of these genes identified the causal mutations in known retinal genes in 11 out of our 21 retinal detachment families and 2 out of our 16 RP families. Thus, 35% of the patients were successfully genotyped in our study with six novel and seven previously reported mutations in known genes.

Interestingly, 65% of our cohort of arRP and NCRNA patients remains genetically undetermined, despite our intensive search. After our prescreening for known mutations in NCRNA and RP genes using a combination of conventional genotyping methods, the genetic defects in 10 NCRNA and 14 RP patients

remained unexplained suggesting new gene(s) in those patients. We therefore subsequently utilized our recently developed RetNet chip screening (where we include 243 retinal dystrophy genes provided in the Retinal Information Network, https://sph.uth.edu/retnet/sum-dis.htm#A-genes). Patients that are negative for this screening phase (thus do not have any mutations in any of the currently known retinal disease genes) are subjected to whole exome sequencing and next generation sequencing (NGS) (Illumina HiSeq 2000) creating a list of exciting and new candidate genes for those two groups of diseases.

Although the clinical diagnosis of all children was NCRNA, in eight families, the molecular diagnosis determined that the disease process was in fact, familial exudative vitreoretinopathy (FEVR), a rare inherited vitreoretinal dystrophy characterized by the disruption of retinal vascular development (FEVR; MIM 133780), caused by pathogenic variation occurred in *LRP5*, *TSPAN12* and *NDP* genes. However, the phenotype remains the very severe congenital retinal detachment, previously not known to be associated with FEVR mutations. Therefore, we have expanded the phenotypic spectrum of FEVR, a severe retinal detachment phenotype that clinically overlaps with NCRNA. Also, we identified in our Pakistani cohort, the previously identified large deletion (6523 bp del) in *ATOH7* found in the Kurdish founder population of Northern Iranian with a high incidence of NCRNA (Ghiasvand *et. al*, 2011) [5]. We therefore establish for the first time genetic overlap between the Iranian and Pakistani populations.

In summary, we have utilized a novel 4-step genotyping protocol, which allowed us to identify novel mutations in known genes with a new phenotype, a new link between the Kurdish population of Iran and the Northern population of Pakistan.

Most exciting is that we have ruled out mutations in all currently known retinal disease genes in a significant number of patients, which likely harbor mutations in new genes. New genes lead to new disease mechanisms and insight into retinal development, retinal biology and possible treatment avenues.

RÉSUMÉ

La cécité infantile causée par des maladies héréditaires de la rétine représente un énorme problème clinique. La morbidité, la cécité mais aussi le manque important de la compréhension des événements moléculaires génétiques conduisant à la cécité chez les très jeunes enfants diminuent la capacité de gérer cliniquement ce problème important et commun. La cécité est une maladie multifactorielle avec des composants génétiques, environnementaux, optiques, développementaux et oculaires. Un moyen de comprendre le mécanisme de la maladie de la cécité infantile est d'étudier les maladies mendéliennes causées par des liens relativement simples entre les mutations génétiques, qui rendent aberrant le fonctionnement des protéines rétiniennes, et le phénotype de la maladie qui en résulte. Ces découvertes dans le modèle mendélien conduiraient à la compréhension des mécanismes des maladies rétiniennes ainsi que d'autres maladies plus complexes provoquant la cécité. Cela produirait de l'information clinique pertinente pour guider les ophtalmologistes, les scientifiques et les patients.

Selon l'Organisation mondiale de la santé (OMS), il y a environ 1,5 million d'enfants aveugles dans le monde dont les deux tiers résident en Asie. La cécité infantile est 66% héréditaire, ce qui le fait le plus grand sous-groupe. Au Moyen-Orient, au Pakistan et dans d'autres sociétés où le mariage consanguins sont commun, la cécité est relativement plus fréquente [1-4].

En collaboration avec un groupe de généticiens, ophtalmologues et les boursiers

de Islamabad et Pakistan où les mariages consanguins sont également fréquents, nous avons rassemblé une collection très intéressante de 21 pedigrees consanguins dont les membres possèdent une maladie autosomique récessif aux effets dévastatrices: le détachement rétinien congénital, aussi connu sous le nom nonsyndromic congenital retinal nonattachement (NCRNA) ainsi que 16 familles avec rétinite pigmentaire autosomique récessive (arRP). Nous avons vérifié les phénotypes, obtenu les autorisations scientifiques et éthiques, signé le consentement et recueilli l'ADN de ces familles pour procéder à notre analyse génétique.

Notre principale hypothèse de base est que une partie importante de NCRNA sera causée par des mutations de nouveaux gènes rétiniennes puisque actuellement un seul gène NCRNA est connu (*ATOH7*). Ces nouveaux gènes peuvent nous donner des informations sur la mécanisme de la maladie de la détachement rétinienne. Décollement de la rétine chez les adultes sont fréquents et les voies de la maladie sont inconnues. Dans une étude préliminaire de notre cohorte, nous avons effectué de génotypage de SNPs où nous avons cherché à identifier des régions homozygotes (hmz). Les régions homozygotes contiennent le gène responsable dans des familles consanguines. Nous avons constaté que, parmi beaucoup de nos patients, les gènes de la détachement rétinienne ou de RP résidaient dans l'un des 5 plus grands secteurs homozygotes documentés par l'ensemble SNP. Le séquençage direct de ces gènes nous a permis d'identifier les mutations responsables dans les gènes rétiniens connus pour 11 sur nos 21 familles de décollement de la rétine et 2 sur nos 16 familles de RP. Conséquemment, six nouvelles mutations et sept mutations rapportées dans des gènes connus se

trouvent dans 35% des patients.

Fait intéressant, 65% de notre cohorte de patients arRP et NCRNA demeurent génétiquement indéterminée, en dépit de notre recherche intensive. Après notre présélection pour les mutations connues dans les gènes NCRNA et RP en utilisant une combinaison de méthodes de génotypage conventionnelles, les défauts génétiques dans 10 patients NCRNA et 14 patients RP sont restées inexpliqués suggérant de nouveaux gène(s) chez ces patients. Conséquemment, Nous avons eu recours à notre puce RetNet (contenant 243 gènes pour la dystrophie rétinienne obtenue par le Réseau d'information sur la rétine, https://sph.uth.edu/retnet/sum-dis.htm # A-gènes) pour continuer la sélection. Les patients qui ont eu un résultat négatifs pour cette phase de sélection (donc qui n'auront pas de mutations dans l'un des gènes connus de maladies rétiniennes) subiront le séquençage complet de l'exome ainsi que le séquençage de prochaine génération (NGS) (Illumina HiSeq 2000) créant une liste de nouveaux gènes candidats pour ces maladies a été formulée.

Bien que le diagnostic clinique de tous les enfants était NCRNA, dans huit familles, le diagnostic moléculaire a déterminé que le processus de la maladie était en fait familial exudative vitreoretinopathy FEVR, une rare dystrophie vitréo-rétinienne héréditaire caractérisée par la perturbation du développement vasculaire rétinienne (FEVR; MIM 133780), causé par des variations pathologiques survenues dans les gènes *LRP5*, *TSPAN12* et *NDP*. Cependant, le phénotype reste d'être des détachements de la rétine congénitale sévères qui n'étaient pas connue pour être associée à des mutations FEVR. Par conséquent, nous avons élargi le spectre phénotypique de la FEVR, un phénotype de

détachement rétinien sévère contenant des caractéristiques cliniques similaires à NCRNA. En outre, Nous avons trouvé, dans notre cohorte pakistanaise, la suppression de grande taille (6523 pb del) dans *ATOH7* préalablement identifiée parmi la population fondatrice kurde du nord de l'Iran chez qui on retrouve une incidence élevée de NCRNA (Ghiasvand *et. al.*, 2011). Nous avons donc établi, pour la première fois, le chevauchement génétique entre les populations iraniennes et pakistanaises.

En résumé, nous avons utilisé un nouveau protocole de génotypage en 4 étapes qui nous a permis de faire lien entre de nouvelles mutations dans des gènes connus et un phénotype nouveau. Nous avons aussi trouvé un nouveau lien entre la population kurde d'Iran et de la population du Nord du Pakistan. La nouvelle la plus excitante, par contre, est que nous avons pu écarté les mutations dans toutes les gènes connus de maladies rétiniennes dans un nombre significatif de patients, qui possèdent en eux fort probablement des mutations dans des nouveaux gènes. Ces derniers nous permettront de construire de nouveaux hypothèses sur le mécanisme de la maladie et du développement de la rétine, ouvrant ainsi de nombr eux avenues de traitement possibles.

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Genes

KITLG	Homo sapiens KIT ligand
BCAR1	Breast cancer anti-estrogen resistance 1
COL4A2	Collagen, type IV, alpha 2
FAM111B	Family with sequence similarity 111, member B
SIRPA	Signal-regulatory protein alpha
RHOD	Ras homolog gene family, member D
NYX	Nyctalopin
CACNA1F	Calcium channel, voltage-dependent, L type, alpha 1F subunit
OVOL1	Ovo-like 1
ZNF408	Zinc finger protein 408
NDP	Norrie disease gene
FZD4	Frizzled family receptor 4
LRP5	Low density lipoprotein receptor-related protein)
TSPAN12	Transmembrane 4 superfamily member 12
ABCA4	ATP-binding cassette, sub-family A member 4
ELOVL	Fatty acid elongase 4
RHO	Rhodopsin
GNAT1	Guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1
PDE6B	Phosphodiesterase 6B, cGMP-specific, rod, beta

Abbreviations

Chr.	Chromosome
Fam.	Family
IBD	Identity by Decent
LOH	Loss of heterozygosity
NGS	Next Generation Sequencing
hmz	Homozygous
IRD	Inherited retinal dystrophy
RP	Retinitis Pigmentosa
NCRNA	Non-Syndromic congenital retinal non-attachment
RNC1-21	NCRNA family label
RP1-16	RP family label
ar	Autosomal Recessive
AC	Anterior Chamber
BDGP	Berkeley Drosophila Genome Project
CSNB	Congenital stationary night blindness
FEVR	Familial exudative vitreoretinopathy
ad	Autosomal dominant
LCA	Leber congenital amaurosis
ERG	Electroretinogram
RPE	Retinal pigment epithelium
GDP	Guanosinediphosphate
GTP	Guanosine-5'-triphosphate

cGMP	Cyclic guanosine monophosphate
PDE	Phosphodiesterase
CNG	Cyclic nucleotide gated channels
Rh	Rhodopsin
OS	Outer segment
ER	Endoplasmic reticulum
IS	Inner segments
SNP	Single nucleotide polymorphism

CHAPTER 1: GENERAL INTRODUCTION

1. Introduction

Inherited retinal dystrophies with photoreceptor death lead to blindness in millions of people including millions of children. The genetic and phenotypic heterogeneity is striking and hampers rapid gene identification. Currently more than 50 mutant genes have been identified just for retinitis pigmentosa (RP) alone [6], 19 mutant genes associated with the congenital form of RP, namely Leber congenital amaurosis (LCA) and one mutant gene for nonsyndromic congenital retinal detachment (NCRNA) [5]. However only ~50% of new RP and LCA patients can be successfully diagnosed at present, indicating that a large number of new genes still await discovery. Gene identification leads to immediate relief for the patients and families, as it confirms the diagnosis, allows for prognostic counseling with respect to future vision and progressive decline estimates, and allows for immediate genetic counseling. New genes also offer hope for scientists, as they point to disease pathways and previously unknown disease mechanisms that may lead to new treatment insights. For basic science, identifying new genes leads to protein network identification and understanding of new disease pathways, new disease mechanisms and insights into photoreceptor biology.

Previously, these diseases were thought to be incurable, therefore the most exciting aspect of this field is the fact that some patients with RP and LCA have been successfully treated with intraocular gene or drug therapy leading to recovery of vision [7, 8, 9]. These exciting studies form the backdrop to our gene discovery studies, and illustrate both the necessity and benefit of not only finding

all the retinal dystrophy genes but also finding them as fast as possible, because the retinas are dying, patients are losing vision and clinical trials are here. Each patient where the causative mutant gene is identified becomes a potential candidate for future therapy.

Photoreceptors are modified cilia that provide vision through a complicated, genetically determined program. Death of these light-sensing photoreceptor cells causes irreversible blindness in inherited retinal degeneration (IRD), the most common cause of visual loss in the world, which affects more than 16 million patients [10]. Loss of work, unemployment subsidies and blindness services cost many millions of dollars. The genes that operate in the genetic program that controls vision are only partially known and understood. Inherited retinal degenerations result from mutations in over 200 retinal genes that cause photoreceptor death (RetNet), explaining approximately 50% of cases, while the remaining genes underlying the residual 50% of patients await discovery and forms one of the goals of this MSc project.

In collaboration with a group of geneticists, ophthalmologists and fellows in Islamabad, Pakistan, we have assembled a very exciting collaboration which led to the collection of 21 consanguineous pedigrees with a devastating Mendelian disease, nonsyndromic congenital retinal detachment, also known as NCRNA and 16 families with RP. We collected the phenotypes, obtained the scientific and ethical approvals, signed the consents, collected the DNA on these families and analyzed them. Pakistan is a place with high incidence of consanguinity and inbreeding which make this population ideal for both population genetic studies and finding novel genes and new mutations. We have therefore chosen the

Pakistani population as the basis for my MSc thesis, and my gene-identification work.

2. Basic anatomy of the eye

As a highly complex structure, the human eye is designed to help gather all visual information about the environment around us and send this information to the visual cortex in the brain to perceive, understand and interpret it. As a complex sensory organ the eye plays an important role in our daily life and understanding the basic anatomy of the human eye would help us to perceive the diseases related to it in a better way.

A cross-sectional view of the eye comprised of three different layers and three chambers of fluid. The external layer consisted of the sclera and cornea. The intermediate layer formed by two parts, anterior (iris and ciliary body) and posterior (choroid) layers, and the internal layer is the retina, which is called the sensory part of the eye (Figure1). The iris is a colored circular muscle and pupil is located in the center of the iris and helps light to enter to the eye. The cornea, a transparent external layer covers both the pupil and the iris. Together with the lenses (which is located behind the iris and can be seen only in sagittal section of the eye) cornea produces a sharp image at the retinal photoreceptor level. Surrounding wall of the eyeball is called the sclera, which is continuous with the cornea [11].

Three chambers of fluid include anterior chamber (located between cornea and iris), posterior chamber (located between iris, zonule fibers and lens) and the vitreous chamber (located between the lens and the retina). The anterior (AC) and

posterior chambers (PC) both are filled with aqueous humor and the vitreous chamber is filled with the vitreous humor, which is a more viscous fluid. Accommodated images are focused in the central point of the retina called fovea. Fovea helps the resolution of the finest detail of the image and transmits it together with the information from the extra foveal and peripheral retina to the brain through the optic nerve [11].

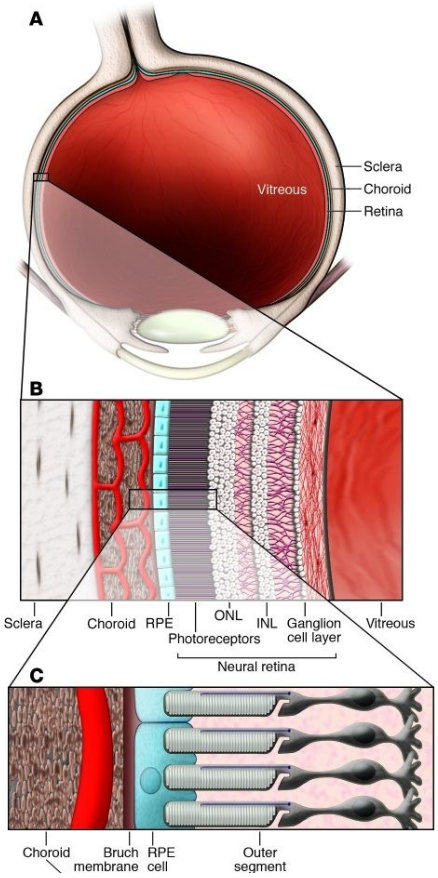


Figure 1. Eye anatomy.

(A) Cross-sectional view of the eye. (B) Cross-section of the retina. INL, inner nuclear layer; ONL, outer nuclear layer. (C) Different layers of retinal tissues (from Alan *et. al.,* 2010).

3. The simple structure of the retina

The thin, highly complex neural tissue lining in the back of the eye is called the retina. The retina is an out-pouching of the brain and this neural component of the eye is the most important layer of the eye for visual perception. The retina is the first place where absorbed light is processed as visual information. It is considered the most metabolically active tissue in the body; the consumption of oxygen per gram is the highest compared with the other organs in the body and the high proportion of this amount is used by photoreceptors. The retina supports this high metabolic activity by two separate vascular beds. It is because of this delicate balance that any disruption that may occur in retinal vascular development will result in severe visual impairments, such as neovascularisation which eventually leads to intraocular hemorrhage, scarring, and retraction of the retina. For example, retinal hypovascularization observed in the human disease called familial exudative vitreoretinopathy (FEVR) and osteoporosis-pseudoglioma syndrome (OPPG) is the result of disruption in retinal vascular development [12].

The retina is comprised of a supporting layer, the retinal pigment epithelium (RPE) which is a single cell layer supporting the delicate membrane of 10 neural layers of specialized neural cells. The RPE separates the neural retina from the choroidal vessels, and supports and nourishes the neural retina cells (Figure2A) [13]. The neural retina has a complex structure and is composed of neuronal cells interconnected by synapses (Figure2B).

Both cone and rod photoreceptor neurons consist of an outer segment (OS) and the inner segment (IS). The OS is connected to the IS, which are connected to the

nuclear body, and the synaptic terminus. The connecting cilium consists of stacks of membranous discs that contain opsin and 11 cis retinal, together forming rhodopsin and the cone pigments, the molecules that absorb photons of light [14, 15]. The inner segment is known as a site for protein synthesis and metabolic processes.

Arguably, the most important cells in the retina are these specialized photo-sensor neurons, the photoreceptors that help the eye transform incoming photons of light into neural impulses to the visual cortex in the brain. Photoreceptors lie outermost in the retina against the retinal pigment epithelium (RPE) and choroid. A human eye has two types of photoreceptors, rods and cones, which are morphologically and functionally very different from each other; rods are cylindrical elongated sensitive structures, responsible for vision in dim light [16] and can respond to even a single photon of light [15, 16], whereas cones mediate vision in bright light and are responsible for color vision, and the perception of fine details. Three types of cones are seen in humans which respond to three different spectra; short-wave length (426 nm) or blue light, middle-wave length (530 nm) or green light and long-wave length (560 nm) or red light [17]. The cones are predominantly located in foveal retina and whereas rods count are dominant in peripheral retina [18]. Although the human retina contains only about 6 million cones compared to 120 million rod photoreceptors, sharpness in vision relies upon the use of cone photoreceptors [19].

Retinal bipolar cells are sensitive to detect the electrical signals coming from the hyperpolarized photoreceptor membranes from both rods and cones. These signals are processed and modified by horizontal and amacrine cells. Modified

electrical signals are assembled by retinal ganglion cells (RGC) located in innermost of the retina and these signals are transmitted to the visual cortex in the posterior brain with the help of optic nerve located in the center of the retina and composed of the RGC axons [17, 18]. The major blood vessels of the inner retina are spreading out from the center of the optic nerve [18]. The outer retina is supplied by the blood vessels of the choroid, underneath the RPE layer.

The central region of the retina known as the macula and in the center of the macula there is blood vessel-free reddish spot, which is the most cone-rich region of the retina (Figure2A) called the fovea and enables high visual acuity in humans [16].

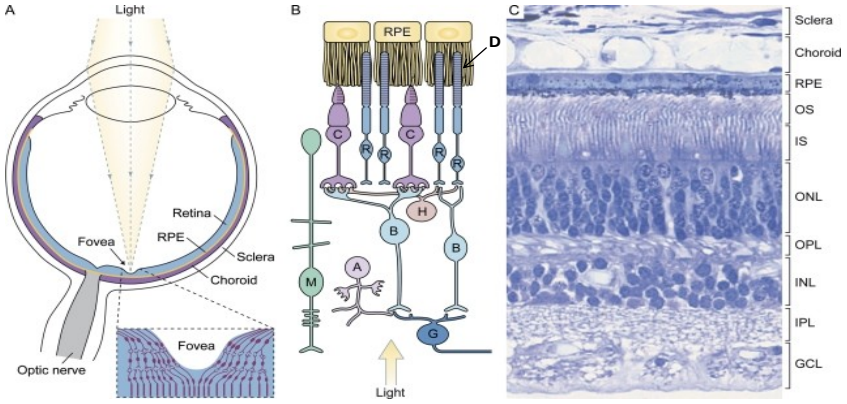


Figure 2. The visual sense organ.

(A) Diagrams of the eye; Retina is located in the posterior part of the eye in a tight relationship with the RPE layer. (B) Structure of the retina; diagram of the organization of retinal cells. A, amacrine cell; B, bipolar cell; C, cone photoreceptor cell; D, the stacked discs of photoreceptor cells; G, ganglion cell; H, horizontal cell; M, Müller cell; R, rod photoreceptor cell. (C) Stained sections of the retina. The outer nuclear layer (ONL) comprised of the nuclei of the photoreceptors. The nuclei of the bipolar cells, amacrine cells, horizontal cells, and Müller glial cells together form the inner nuclear layer (INL), whereas the ganglion cell layer (GCL) contains the nuclei of ganglion cells. The processes and synaptic terminals of photoreceptors, horizontal cells, and bipolar cells create the outer plexiform layer (OPL) and the processes and terminals of bipolar cells, amacrine cells, and ganglion cells build up the inner plexiform layer (IPL). The outer segment (OS) of rod photoreceptor cells is tightly attached to RPE layer. (from Sung *et. al*. 2010 [16]).

4. Molecular mechanisms of vision

Opsins are light-sensitive pigments, which are located in the outer segment (OS) of rod photoreceptor cells absorbing a photon when the light hits the eye [17]. They are able to convert the light into an electrical signal, which creates the visual cycle [17, 20]. Rhodopsin (Rh) is a protein-chromophore complex (Rh) embedded in the rod outer segment disc membranes [21] and composed of the opsin protein, and a light sensitive chromophore 11-cis-retinal [22]. Opsin is synthesized in the inner segments (IS) of photoreceptors, glycosylated in the endoplasmic reticulum (ER), further modified in the Golgi membranes and transported to the OS with the help of connecting cilium where it is inserted into the disc membranes [23].

Vision occurs when rhodopsin absorbs a photon that isomerizes a derivative of vitamin A, 11-*cis*-retinal to all *trans*-retinal initiating the activation of phototransduction cascade and catalyzing the conversion of inactive GDP-bound transducin to its active GTP-bound conformation (Figure3A). The photo-activated rhodopsin catalyzes the conversion of inactive GDP-bound transducin to its active GTP-bound conformation. In turn membrane-associated phosphodiesterase (PDE) is activated which hydrolyzes cyclic guanosine monophosphate (cGMP). In the dark, cGMP is bound to cyclic nucleotide gated channels (CNG) that responsible for the influx of cations (Na+, Mg2+, Ca2+) through the photoreceptor plasma membrane allowing a steady inward current [24, 25].

This dark current keeps the photoreceptor interior depolarized with a resting membrane potential of -40mV. Reduced intracellular cGMP (as a result of the capture of a photon of light) leads to closure of the cation channels and a block in the cationin flux (Figure3). The resulting hyperpolarized membrane of the

photoreceptor inhibits the release of glutamate to the connected bipolar cells and provokes the adjacent neurons. The electrical signals from the hyperpolarized photoreceptor membrane are transmitted to the visual cortex of the brain by the retinal ganglion cell axons [17, 25].

In the light, the phototransduction cascade leads to closure of the channels and hyperpolarization of the neuronal membranes, in parallel, photoisomerization takes place leading to a second process called the visual cycle or retinoid cycle (Figure3B), which is essentially a cycle to replenish the critical compound 11 cis retinal, the only available molecule to capture photons of light. When a photon of light strikes visual pigments, it converts the 11-*cis* to all- *trans* retinol configuration. Following this, another change leads to formation of all-trans retinol. All-trans retinol is transported to the RPE and then it is regenerated back into 11-*cis*-retinal by a series of enzymatic steps [24, 25, 26].

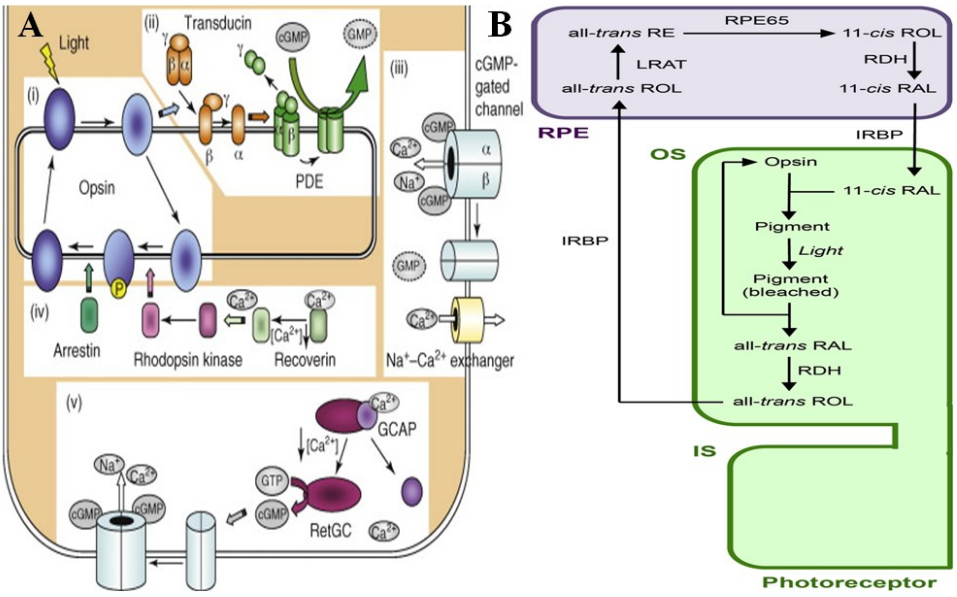


Figure 3.Visual cycle including phototransduction and the retinoid cycle.

A. The phototransduction cascade. In the dark current, the cGMP-gated channels are open, this leads the entry of ions and depolarisation of the cell; When light strikes the opsin the retinoid changes conformation (i) opsin activate transducin (ii) transducin activates cGMP-phosphodiesterase (PDE) which in turn hydrolyses cGMP (iii) the cGMP-gated channels are closed and the cell hyperpolarizes (iv) a signal is send to the neurons in the inner retina (v). Decrease in the Ca^{2+} concentration leads to the activation of recoverin (vi). Recoverin in turn activates rhodopsin kinase, and phosphorylates (P) the opsin. The reduction of Ca^{2+} concentration also causes activation of retinal guanylatecyclase (RetGC) (vii). cGMP concentration is increased by RetGC, this opens the cGMP-gated channels, enabling influx of ions, depolarizing the cell and returning it to the dark current (viii) (from Smith *et. al.* 2009). **B.** The retinoid cycle illustrating regeneration of 11-*cis*-retinal which occurs in rod photoreceptor cells and the retinal pigment epithelium (RPE). Retinal (RAL); retinol (ROL); retinol dehydrogenase (RDH); lecithin retinol acyltransferase (LRAT); retinyl-ester (RE) (from Tang *et.al*., 2013) [24].

5. Inherited retinal dystrophies overview

A genetically heterogeneous group of hereditary diseases associated with visual loss and blindness are grouped under the heading inherited retinal dystrophies (IRD). These disorders are progressive and cause vision loss during childhood. The IRD can be stationary or progressive and all modes of inheritance are observed. They are characterized by degeneration of the photoreceptor and the RPE cells, and can be nonsyndromic which is restricted to the eye or syndromic accompanied by disorders of other organs such as deafness (Usher syndrome) or polydactyly/developmental delay (Bardet Biedl syndrome). They include retinitis pigmentosa (RP) and Leber congenital amaurosis (LCA), Stargardt's disease, familial exudative vitreoretinopathy (FEVR), non-syndromic congenital retinal non-attachment (NCRNA), congenital stationary night blindness (CSNB), osteoporosis-pseudoglioma syndrome among the others.

According to the study conducted by Tabarra *et. al.* (1985) from 1962 onwards to recently, 84% of childhood blindness in Saudi Arabia was the result of hereditary diseases. 70% of the studied patients were blind before age 2 and the rest before the age of 14 and 56% of these patients were the result of consanguineous marriages [27].

The clinical diagnosis of these disorders is based on various ophthalmological tests (see methods) and genetic testing. In short, these tests measure visual acuity, visual field, retinal sensitivity and *in vivo* retinal architecture.

The discovery of the genes important for retinal dystrophies helps to reveal new biological pathways in retinal structure and function. The first step to understand the mechanism of retinal dysfunction and develop effective treatment is

identification of the genes underlying inherited retinal diseases. Moreover, it is essential for clinical management, diagnosis and genetic counseling.

Over the last few years, more than 200 retinal genes responsible for inherited retinal diseases have been identified (Figure4) (see RetNet web site: http://www.sph.uth.tmc.edu/RetNet/). This amount of genetic and allelic heterogeneity was unexpected and is unprecedented in human disease. These genes help to determine the molecular defect underlying IRDs in up to 50% of cases [28] (approximately 50% of AR RP, 30% of LCA, 60% of AR CRD, and 90% of AR CD undiscovered [26]) currently, indicating that additional genes responsible for these conditions are still unrevealed.

A definitive diagnosis with genetic testing and regular follow-up is essential and beneficial for future therapies and would help to adjust to a future that includes visual disability as the loss of vision due to inherited retinal disease affects people's life significantly and sometimes devastating it in different ways.

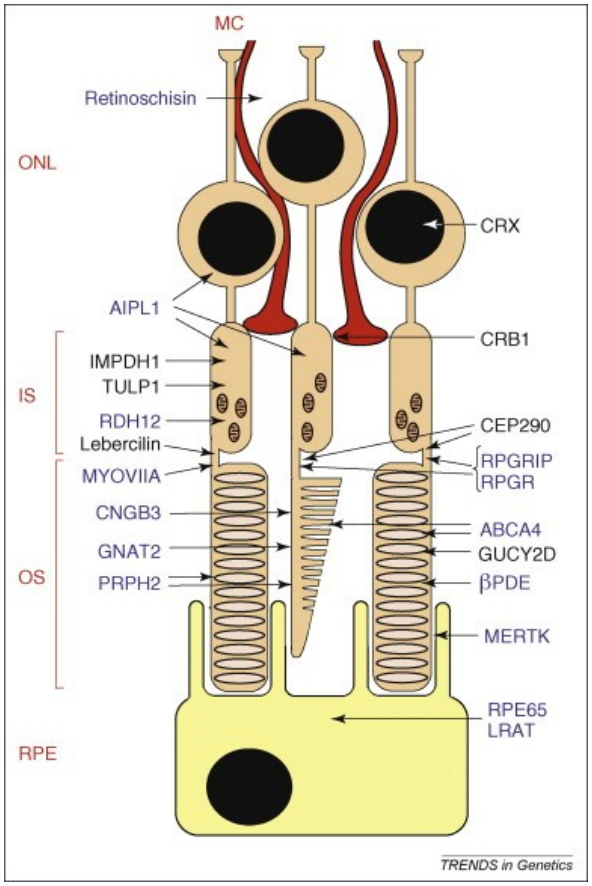


Figure 4. Localization of the proteins in early-onset retinal dystrophies.

Inner Segments (IS) or the outer segments (OS) of the photoreceptor cells are carrying the most of the proteins responsible for early-onset retinal dystrophy. Müller cell (MC) processes are shown in red. *RPE65*, *ABCA4*, *LRAT* and *RDH12* are the genes that play role in visual cycle. *GNAT2* and *CNGB3* play role in cone phototransduction and βPDE in rod phototransduction. Ciliary proteins, RPGRIP, RPGR and MYOVIIA, are involved in the transport to the outer segment. *PRPH2* and Retinoschisin are structural proteins. RPE, retinal pigment epithelium; ONL, outer nuclear layer (from Smith *et. al.* 2009).

5.1. Retinitis pigmentosa

Retinitis pigmentosa (RP) is a group of inherited genetic retinal dystrophies that cause slow but progressive retinal degeneration with photoreceptor loss leading to gradual, permanent and complete blindness. The disease starts in the mid-periphery of the retina and progresses towards the macula and fovea eventually causing central vision loss. RP is a genetically heterogeneous and also has significant phenotypic variation; the same mutation can phenotypically show different disease manifestations in different patients. Night blindness, reduced visual fields leading to tunnel vision (abnormal electroretinogram and attenuated retinal vessels) and pigmentary deposits, called bone spicules (Figure5 and 6), predominantly in the peripheral retina, are typical signs and symptoms [29]. Prevalence of the disease varies and depends on the ethnic background with a worldwide prevalence of 1: 3000 - to 1:7000 people. In most populations the incidence is 1 in 3500 people, which represents a common disease. In isolated ethnic groups this number can reach up to 1:2400 [30]. All types of inheritance; autosomal dominant (adRP), autosomal recessive (arRP), and X linked (XLRP) can be seen in RP. About 50% of RP patients are sporadic with no family history, while 20% are inherited as adRP, 20% as arRP, and the rest (10% of patients) are known to be X-linked [17]. Although the age of onset varies among RP cases, X-linked RP (XLRP) has the earliest onset (5-10 years of age) and considered the most severe type of RP with rapid progression [31]. adRP represents a milder form of the disease compared to arRP.

Usually, the disease is diagnosed in young adulthood, although it can also present in the younger ages. The disease always starts with rod death and degeneration

followed by cone death. The photoreceptor cell death is thought to be by apoptosis as the final common pathway. The disease is genetically heterogeneous and currently ~50 different genes identified that are responsible for the different types of RP (adRP -12 known genes, arRP- 22 different known genes, XLRP- 2 known genes)[6].

RP can be nonsyndromic which is restricted to the eye, or syndromic accompanied by other systemic disorders (especially loss of hearing, Usher syndrome), which occurs in approximately 30% of patients [32].

Phenotypically, optical coherence tomography (OCT), electroretinogram (ERG), Goldmann visual field and visual acuity can be helpful to diagnosis and prognosticate the disease.

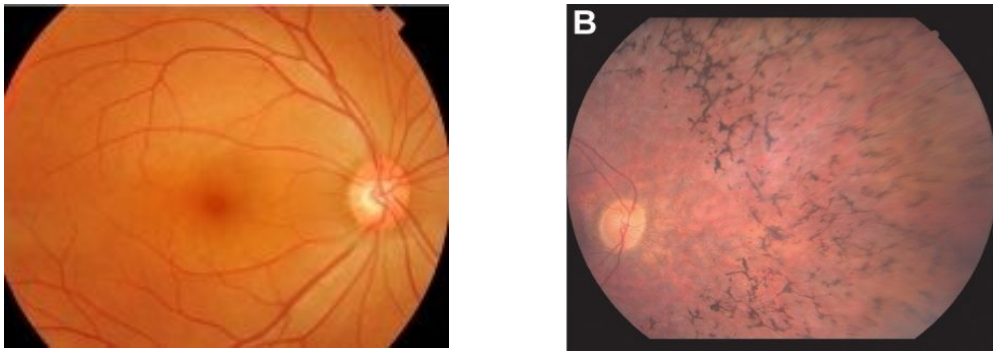


Figure 5. Retinal changes in RP.

Normal human retina (on the left) compared to a fundus photo (on the right) of a RP patient with bone spicules, narrow blood vessels and areas of atrophy (from Bowne *et. al.* 2008).

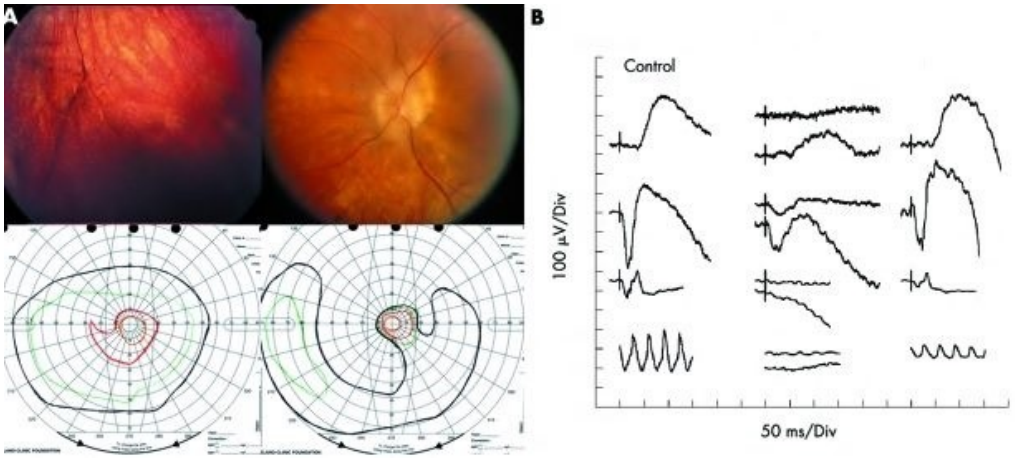


Figure6. Fundus photographs, visual field and ERG records in RP.

(A) Fundus photos with some fine pigmentary changes and bony spicules (top). Bottom panels illustrate visual field defects. (B) ERGs record from a normal control subject (left) and with retinitis pigmentosa (middle). First and second rows are dark adapted ERGs; third, light adapted ERG; fourth, 31 Hz flicker ERG (from Melamud *et. al.* 2006).

5.2. Leber congenital amaurosis

Leber congenital amaurosis (LCA) is a group of early-onset photoreceptor cell degenerations characterized by congenital blindness or severe loss of vision at very young age, usually at around the age of 6 weeks [34],with an incidence of ~1 in 81,000 [33]. It is bilateral retinal dystrophy comprising 5% of the total inherited retinal degenerative diseases in children [34]. LCA is a genetically and clinically heterogeneous autosomal recessive disease (although some autosomal dominant cases were also reported) implicated by mutations in at least 18 [35] different genes, which account for approximately 70% of cases [34]. These genes are involved in variety of functional pathways, including photoreceptor development,

phototransduction, retinoid cycling, ciliary transport, guanine synthesis, and outer segment phagocytosis by the retinal pigment epithelium. A severely reduced or absent rod and cone signals on the electroretinogram (ERG), nystagmus, photophobia, sluggish or near-absent pupillary responses, hyperopia, amaurotic pupils attenuated vessels, atrophy of the retinal pigment epithelium (RPE) and a highly variable retinal appearance are other features of LCA (Figure7).

Although there is an overlap of phenotypic expressions between different genotypes in LCA [34], phenotypic differences in patients with the same genotype introduce difficulties to clinical diagnosis (Figure7).

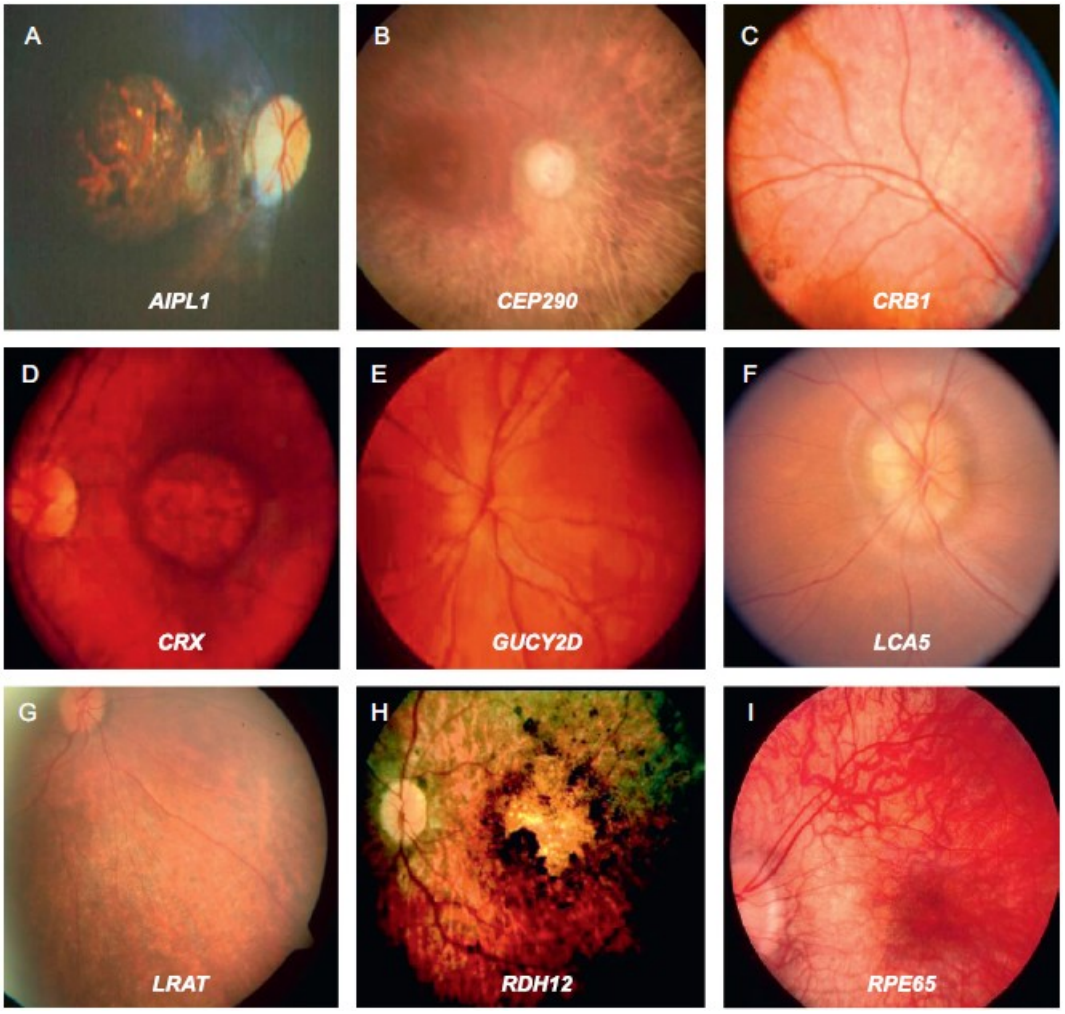


Figure7. Distinct retinal phenotypes in LCA.

Different genes cause distinct retinal phenotypes in LCA (from den Hollander *et. al*., 2008) [34].

5.3. Non-syndromic congenital retinal non-attachment

Non-syndromic congenital retinal non-attachment (NCRNA) is an autosomal recessive, congenital, devastating form of retinal detachment leading to permanent blindness from birth. Children with NCRNA have complete retinal detachment and total blindness from birth and the retina is partially or totally separated from the posterior globe. Main symptoms for NCRNA are congenital insensitivity to light, massive fibrovascular mass behind the lens (Figure8), shallow anterior chamber, microphthalmia, and nystagmus [5]. The disease is clinically and genetically heterogeneous, with a variety of clinical and histological anomalies involving the retina and blood vessels in one or both eyes. In some individuals, the disease occurs with systemic malformations, but there is no consistent association. Recently, sporadic and familial cases have been reported. A nonsyndromic hereditary form (NCRNA) is common in North Khorasan, Iran, among descendants of a Kurdish founder population and it is inherited as an autosomal recessive trait with complete penetrance. The locus was mapped by DNA microsatellite linkage and haplotype analysis to a 0.6–1.5 cM region on chromosome 10q21 [5].

Up to date mutations in only one causal gene, *ATOH7* has been described in the Kurdish population [5]. This means that many more genes underlying NCRNA are likely present, forming the basis of this thesis and research. The previous researchers have revealed that NCRNA may be caused by a 6, 523 bp deletion of conserved DNA sequences of *ATOH7*, a cluster of three conserved noncoding elements that spans a remote cis regulatory element 20 kb upstream from *ATOH7* (also known as *Math5*) [5]. *ATOH7* is a transcription factor gene that is required

for retinal ganglion cell (RGC) and optic nerve development. It was determined that blind individuals with NCRNA lack optic nerves, which prevents light perception and profoundly affects the development of retinal vasculature and the absence of RGCs creates neovascular growth of fetal blood vessels in the vitreous and early retinal detachment. Optic nerve defects have been investigated as a primary anatomical basis for NCRNA pathology. Thus, NCRNA is a Mendelian disorder that disrupts the intrinsic mechanism of retinal cell fate determination and leads to early onset retinal detachment and subsequent blindness [5]. *ATOH7* will be our entry point into finding other NCRNA genes and proteins. Also NCRNA gene discoveries will be important for our hypothesis that mutations in these Mendelian disorders may give us insight into the genes and disease mechanisms of adult retinal detachments, a huge and large unexplained clinical problem.

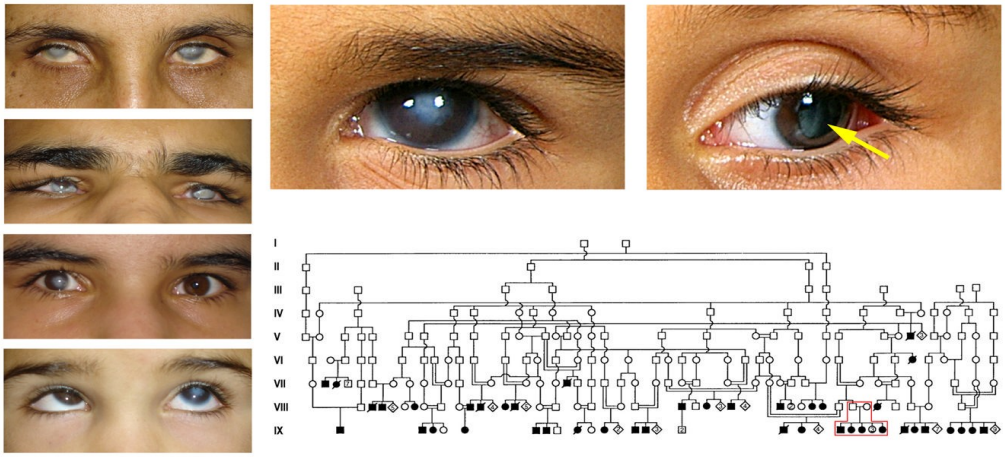


Figure 8. Eye photographs of patients with NCRNA from Khorasani pedigree.

Patients with NCRNA typically exhibit leukocoria (white pupils), posterior corneal opacities consistent with chronic blood staining. The arrow shows the detached retina and fibrovascular mass visible behind the clear lens (from

Ghiasvand *et. al.* 2011) [5].

5.4. Familial exudative vitreoretinopathy

Familial exudative vitreoretinopathy (FEVR) is a rare hereditary disorder caused by incomplete vascularisation of the retina (FEVR; MIM 133780) which may be compatible with normal functioning retina, but may also lead to various complications, such as retinal neovascularisation and exudates, vitreous hemorrhage, vitreoretinal traction with deformation of the posterior retina, ectopia of the macula, retinal fold, tears, and retinal detachment [36]. Highly variable phenotypic variability ranging from normal and no visual impairment to severe vision loss is seen individuals with FEVR. Variability can be observed even between the two eyes of the same individual. Children may become blind shortly after birth in severe form, while in the more common mild form individuals are completely asymptomatic [36, 37].

Although dominant adFEVR is the most common [36], all three kinds of inheritance pattern, ad, ar and X-linked are observed in FEVR, confirming that it is a genetically heterogeneous disease. Thusfar pathogenic mutations in four genes, Norrie disease gene (*NDP*; MIM 300658, X-linked), Frizzled family receptor 4 (*FZD4*, MIM 604579, dominant), low density lipoprotein receptor-related protein **(***LRP5*, MIM 603506, dominant and recessive), and transmembrane 4 super family member 12 (*TSPAN12*, MIM 613138, dominant), encoding components of a novel ligand-receptor complex that play a key role in the Norrin-b-catenin signaling pathway have been identified underlying the disease [38-42]. Mutations in *NDP* are known to be responsible for an X-linked

form of FEVR. *FZD4* has been associated with adFEVR and sequence variants identified in *LRP5* and *TSPAN12* are known to be involved in both, autosomal recessive [38] and adFEVR [36] (*LRP5*; MIM 603506). However, mutations in these genes account only for approximately 50% of cases [43] and it is important to diagnose the disease as early as possible to prevent serious complications in children, which usually take place in the first two decades of their life [36].

The proteins are encoded by these genes play an important role in the Norrin/b-catenin signaling pathway. *FZD4* (Wnt family receptor) and *LRP5* (Wnt coreceptor) both play central roles in the evolutionarily conserved canonical Wnt signaling pathway. Norrin, a protein encoded by the *NDP* gene control vascularization of the retina during development as a direct ligand for the frizzled-4/Lrp5 complex, a component of the canonical Wnt signaling pathway [44, 46]. Wnt signaling is not activated in the absence of Norrin binding and result in repression of Norrin target genes [43]. It is assumed that TSPAN12 binds to Fz4 and stimulates Norrin/Fz4/Lrp5 signaling, presumably by inducing receptor clustering [45]. Thus, only co-expression of Norrin, Frizzled-4, LRP5 and TSPAN12 activates Wnt signaling [45, 46]. Taking into consideration all information presented above, it is clear that identifying new mutation in these genes and new FEVR genes will assist to broaden knowledge about this important signaling pathway which may eventually lead to successful treatments.

5.5. Congenital stationary night blindness

Congenital stationary night blindness (CSNB) is a group of genetically heterogeneous, non progressive retinal diseases that starts in early childhood and

is characterized by poor night vision and differs significantly from RP in that there is no apparent rod or cone photoreceptor degeneration. Other kind of ocular symptoms are observed also, such as decreased visual acuity, myopia, hyperopia, and nystagmus [47]. All three modes of inheritance, autosomal dominant, autosomal recessive and X-linked can be observed among patients [48]. X-linked inheritance of CSNB is more common which is subdivided into two groups, complete and incomplete, based on the presence or absence of a scotopic b-wave amplitude which are caused by variations in two different genes, *NYX* and *CACNA1F*. The sub groups are distinguished by electroretinogram; in incomplete CSNB, a rod-specific ERG is present and cone ERGs are not recordable, whereas in complete CSNB, the rod-specific ERG is absent [47, 49]. AR CSNB is associated with mutations in *GRM6, TRPM1* and *CABP4* genes. ERG records in adCSNB can also be in consistent with rod system dysfunction while rods show normal ERGs but in some cases rod responses are slightly abnormal with normal cone responses [50]. The genes that encode 3 components of the rod-specific phototransduction cascade, *RHO*, *GNAT1*, and *PDE6B* are reported in association with adCSNB [52, 52]. However, all discovered genes associated with CSNB accounts only approximately 80% of disease [53, 54].

5.6. Stargardt's disease

Stargardt disease is a very severe form of juvenile hereditary macular degeneration, which appears at an early age and is characterized by the collection of a substance called lipofuscin in RPE layer and leads to central visual loss. The disease mostly affects the macular area, which in turn affects central vision [55].

Pisciform-shaped yellow deposits (or flecks) around the macula are common in early stage funduscopic findings, however, a beaten-bronze sheen macular manifestation, macular granularity, a bull's-eye lesion, or retinal pigment epithelium depigmentation with atrophy can be observed in later stages [56, 57]. Symptoms of the disease usually occur during the first and second decades of life but it can be seen in late adulthood also [56]. The disease is inherited in both autosomal recessive (99% 0f cases) and autosomal dominant pattern and it is considered the most common hereditary macular dystrophy with the prevalence of 1 in 8000, 1 in 10,000 [55, 57]. AdStargardt is called a "Stargardtlike" disease, and differs from autosomal recessive form with the mechanism and the gene involved in the disease. The gene responsible for autosomal recessive Stargardt disease is ATP-binding cassette, sub-family A member 4 (*ABCA4*) while ELOVL fatty acid elongase 4 (*ELOVL4*) mutations have been identified as the cause of the very rare autosomal dominant form of Stargardt-like disease [57, 58, 59]. The disease is typically diagnosed by loss of visual acuity, color testing, and visual field, fundus photographs, ERG, and fluorescein angiogram. One of the best methods to describe the severity of the disease is full-field electroretinography [60].

6. Objectives and hypotheses

6.1. Objectives

The goals of this thesis are 1) to identify the genetic causes of autosomal recessive nonsyndromic retinal dystrophies, namely the genes responsible for non-syndromic congenital retinal non-attachment (NCRNA) and autosomal recessive retinitis pigmentosa in the Pakistani population. 2) Improve the clinical management of retinal detachment and retinitis pigmentosa through understanding the molecular events that lead to these two disease groups; 3) to test and validate a new gene-discovery protocol established by our laboratory.

6.2. Hypotheses

Our hypotheses are that 1) finding the gene(s) for NCRNA will allow us to identify the disease mechanism and disease pathways of retinal detachment and get entry into the much more common and complex adult retinal detachment for which no gene or disease mechanism are currently known; 2) the causal gene for RP or NCRNA with mutations lies in one of top five largest intervals of our identified homozygous regions in consanguineous families; 3) a new causal gene underlying the disease may locate in the overlapping homozygous regions of different family members with the same disease phenotype.

6.3. Experimental approach and expectations

Genome-wide high-density SNP genotyping was performed for selected members of each family. Homozygosity based linkage analysis was performed to the known and new gene/s associated with the disease. Sanger sequencing and/or whole Exome sequencing (WES) by next generation sequencing (NGS) was performed to pinpoint the genetic variants responsible for the disease.

Consanguineous families have homozygous regions, one of which contains the causal gene with homozygous mutations. This fact allows for a genetic strategy that is well established, called homozygosity mapping and inheritance by descent (IBD) studies. Our approach to finding the causal genes in NCRNA and RP was to first identify all the significant homozygous regions in the probands and then look for overlap of all these regions with all affected members of that family, followed by searching for overlap with other families. This 3 step process allows us to narrow and decrease the number of homozygous regions. The remaining overlapping regions can then be probed for candidate genes that are expressed in retina or in RPE, using available data bases.

We performed homozygosity mapping by genome wide SNP (single nucleotide polymorphism) arrays, searching for significant stretches of SNPs. We use the Illumina SNP arrays and identify significant stretches by P-link, where more than 100 SNPs are homozygous.

Our gene discovery paradigm consisted of 1. detailed phenotyping through the methods shown in material and methods section, followed by 2. genotyping (see methods), followed by 3. confirmation that we have a new gene by finding null mutations, perfect co-segregation, *in silico* analyses predicting the variants to be

damaging by SIFT, Blosum62 and Polyphen-2. We then study conservation of the residue in homologous proteins of lower animals and we verify that the variant has not been reported in the Exome Variant Server (EVS; NHLBI GO Exome Sequencing Project), dbSNP135 or 1000 Genome datasets. Finally, we exclude the newly found variant from normal controls that are culturally matched to the original family.

CHAPTER 2: MATERIALS AND METHODS

1. Study subjects

21 NCRNA families (28 affected members) and 16 arRP families (40 affected members) were recruited, phenotyped and genotyped from Pakistan and included in this study. Informed consents were obtained from all the patients after full explanation of the procedures was provided. Consents were prepared according to guidelines set forth by the Montreal Children's Hospital ethical review board and from the Institutional Review Boards of the participating centers. Entry criteria were as follows. The diagnosis of NCRNA was given to patients with complete retinal detachment from birth and for RP patients who had difficulties with dark adaptation and progressive night blindness in adolescence and loss of mid-peripheral visual field in young adulthood. Almost all RP patients had non detectable or significantly reduced full-field ERG response and clinical history, visual fields, and the appearance of the fundus confirmed the diagnosis. All known mutations in the known NCRNA and RP genes were excluded with Sanger sequencing or with RetNet chip analyses.

2. Ophthalmic evaluations and examinations

All patients were questioned for detailed ocular and visual histories and pedigrees were drawn. Ophthalmic examinations were performed on all affected and at-risk patients, included visual acuity, electroretinograms according to the standards recommended by the International Society for Clinical Electrophysiology of Vision (ISCEV) protocol, Goldmann kinetic perimetry (V4e and I4e test lights),

and fundus photography.

2.1. Measurement of visual acuity

Visual acuity was measured as explained by Wendy Strouse Watt, O.D. (October 2003) [61] using the Snellen eye chart which was created in 1862 by a Dutch Ophthalmologist, Dr. Hermann Snellen. Broken Wheel vision test was used for children or those who cannot read the alphabet and the person being tested must tell which card has the broken wheels on the pictured car. Another type of eye chart that can be used is a picture chart with common pictures of different sizes. Low Vision Chart was used for the people who had severe visual impairment (to measure the smallest letter the chart moved closer). Some patients had vision worse than 20/400 which is common in RP patients, for those patients vision was recorded as count fingers (we ask them to count the fingers at variable distance), hand motion, light perception (LP), or no light perception (NLP) and records were converted to LogMAR with the help of conversion chart and mean was calculated.

2.2. Goldmann visual field (GVF)

The entire visual field of the patients was determined by a common reliable kinetic perimetry called Goldmann perimetry. The test was performed by plotting points along isopters (circles), which are color-coded to the size and intensity of the stimulus used. Lenses were chosen according to the patient's special situation (lens power may be affected by myopia, pseudophakia or dilation). Records were carried out according to patients' responds to the stimuli (I4e for peripheral and I2e for central visual field). The eye that is not being tested was patched. The

stimuli were moved by pantograph handle from the non-seeing area into a seeing area at about 3-5 degrees per second. The test was performed 10 minutes per eye and one eye at a time. Details of the method are described by Dersu *et. al.* (2006) [62].

2.3. Optical coherence tomography

We used optical coherence tomography (OCT), a well established method for estimating the residual retinal architecture *in vivo* and to assess morphology and function of the remaining photoreceptors and retinal pigment epithelium (RPE) in the macula area, as well as to observe the photoreceptor inner/outer segment (IS/OS) junction (Heidelberg Engineering, Heidelberg, Germany). The degree of normal visual function and approximate amount of normally functioning photoreceptors were determined based on the presence or absence of a distinct and continuous IS/OS line in the OCT images. The absence of the IS/OS junction in the OCT images was associated with worst visual acuity and thinner fovea. To evaluate the visual acuity, the central foveal thickness and foveal outer segments lengths were measured manually using the internal program on the OCT images at the thinnest point of the fovea. Patients with the thinner fovea had worst vision due to photoreceptor loss.

2.4. Fundus autofluorescence

Fundus autofluorescence (FAF) measurement was performed to investigate the topographic mapping of lipofuscin distribution in the RPE cells (which is indicator of the degradation of the photoreceptor outer segments) and to test

metabolism in the RPE cells. Pupils were dilated with topical 0.5% tropicamide and 2.5% phenylephrine before the test and FAF images were obtained according to standard procedure (Heidelberg Engineering, Heidelberg, Germany). The presence of parafoveal FAF ring (AF ring), the border between functional and dysfunctional retina, or absence of FAF were carefully studied in the patients to determine if there is RPE atrophy, loss of photoreceptors, or the presence of materials between the RPE and camera that attenuate the FAF signal. A confocal scanning laser ophthalmoscope was used to obtain FAF images. Details of the test can be obtained from Y. Mitamura*, et. al. (*2012) [63]. Interpretation of FAF images was performed as described by Schmitz-Valckenberg *et. al.(*2008) [64].

2.5. Electroretinography

A standard clinical diagnostic procedure called electroretinography (ERG) is invaluable method for precise diagnosis in RP patients and to evaluate the function of the retina as a whole, including functionality of cons and rods of the eyes. During the test 1% tropicamide and 2.5% phenylephrine eye drops were used to dilate patients' pupils and dark and light adapted ERG was obtained in both eyes. We were looking for the general reduction in the amplitude of five ERG responses (rod, maximum, oscillatory, cone and flicker), especially reduction of scotopic rod response, which is the first sign of RP. Scotopic and mesopic results were carefully studied for early detection of RP combined with other signs and symptoms. Electrical responses of the eyes were recorded with a corneal electrode while the retina of an eye was introduced into stimulation,

flashes of light. Detailed explanation of the steps is demonstrated by Parvaresh *et.al.* (2009) [65] and by Hassan-Karimi *et.al.* (2012) [66].

3. METHODS

Blood samples from all available NCRNA and arRP family members were collected at the Department of Pediatric Ophthalmology and Strabismus Al Shifa Trust Eye Hospital, in Rawalpindi, Pakistan. Additional blood from 100 random unrelated healthy control individuals was also collected for determination of allele frequency in the general Pakistani population.

Genomic DNA was extracted from peripheral blood leukocytes with the FlexiGene kit and the QIAamp DNA blood kit, according to the instructions given in the protocol provided by the manufacturer. DNA quantity and quality was verified by spectrophotometer using a Thermo Scientific NanoDrop 1000. Polymerase Chain Reaction (PCR)-sequencing approach was used to identify mutations in candidate genes. Primers were designed by primer3 online program (http://frodo.wi.mit.edu/primer3/) and by ExonPrimer (http://ihg.gsf.de/ihg/ ExonPrimer.html/ provided in the public domain by the Institute for Human Genetics, Technical University of Munich, Germany). To ensure the completeness and quality of the sequences and for detection of potential mutations located in splice sites, minimal distance between primer and exon/intron boundary were selected at least 60 bp when primers were designed. Sequences for the used primers are listed in Table 18.

The HotStarTaq Master Mix kit from QIAGEN was used to perform the

PCR's following the manufacturer's protocol and the purity of the products were verified by gel electrophoresis using 0.8% agarose gel stained with ethidium bromide. PCR products were sequenced by Sanger sequencing at the Genome Quebec Center and the results were analyzed using the Sequencher software program. Identified mutations were analyzed *in silico* by ENSEMBL, Blocks Substitution Matrix (BLOSSUM) analyses, SIFT and Polyphen to determine their significance.

3.1. SNP Microarrays

The Illumina Human 610 Genotyping BeadChip (610,901 SNPs) was performed for genotyping all consanguineous families and families with more than one affected member. QIAamp DNA Mini Kit (Qiagen, Valencia, CA) was used to purify DNA samples for SNP analysis (200 ng DNA was used from each sample). Detection of the fluorescent intensity generated from the beads was performed with the Illumina iScan Reader, and genotypes were visualized using the BeadStudio software package. Details are provided by Leung *et. al*., (2003) [67]. All experiments are carried out according to manufacturer's protocols. Regions of interest (overlap of homozygous regions of sibs and families) were further analyzed using the Illumina Genome Viewer and P-link software. Chromosomal regions were considered as homozygous if they contained at least 100 consecutive homozygous SNPs.

3.2. RetNet chip screening

14 patients diagnosed with NCRNA and 14 RP patient have been screened by our newly developed RetNet chip (Wang *et. al.* in preparation, 2013), which for the first time contains all the currently known retinal disease genes (243) by targeted NGS. The chip helped us to screen all retinal genes listed in Retinal Information Network website and create candidate retinal disease gene list for future study.

3.3. Next generation sequencing (NGS)

We performed whole exome capture by Roche Nimblegen Sequence Capture Arrays to pinpoint the genetic variants responsible for the disease for those patients who did not carry any known RP or NCRNA genes in their LOH regions and for the patients who's all the known genes residing in LOH regions were excluded by Sanger sequencing. All coding and noncoding exons, including surrounding sequences that covered the splice sites were covered by the array design. Sequence capture was performed in accordance with the instructions of the manufacturer (Roche NimbleGen, the Titanium optimized protocol) in the sequencing platform of the McGill University and Genome Quebec Innovation Centre and in Human Genome Sequencing Center, Baylor College of Medicine, Houston, Texas, USA. Details of the protocol are described by Hoischen *et. al.* (2010) [68] and by Wang *et. al.* (2011) [69].

In summary, the target capture and sequencing of 100 bp paired end reads on Illumina HiSeq 2000 was performed according to the manufacturer's protocols with the Agilent V4 exome enrichment kit. The Fastx toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) was used to remove adaptor sequences

and quality trimmed reads. A custom script was used to ensure that only read pairs with both mates present were subsequently used. Reads were aligned to hg19 with the help of BWA 0.5.9 [70] and indel realignment was carried out utilizing the GATK [71]. Duplicate reads were excluded using Picard (http://picard.sourceforge.net/). Coverage of consensus coding sequence (CCDS) bases was evaluated by the GATK. Average mean read depths of bases in consensus coding sequence exons were: 143.88, 124.41, and 126.64 for each sample. Average CCDS bases covered by at least 10 and 20 reads were (95.8, 93.1), (95.5, 92.2), and (95.5, 92.3) respectively. Single nucleotide variants (SNVs) and short insertions and deletions (indels) were called using samtools mpileup for each sample [72] with the extended base alignment quality (BAQ) adjustment (-E), and were then quality filtered. Both Annovar [73] and custom scripts were utilized to annotate whether the variants affected protein coding sequence, and whether they had previously been seen in dbSNP132, the 1000 genomes databases, and the NHLBI GO exomes.

3.4. Polymerase chain reaction

Polymerase Chain Reaction (PCR)-sequencing approach was used to identify mutations in candidate genes. Details of used primers are given in section 3 (methods) and in Table 18.

The HotStar Taq Master Mix kit from QIAGEN was used to perform the PCR's following the manufacturer's protocol. 30µl PCR reaction contained 1µl DNA, 15µl ready MasterMix, 0.5 µl of each primer (Invitrogen), and 13µl water. Some PCR reactions were conducted using 6µl Q solution provided by QIAGEN

to get clean band for sequencing. The thermal cycling conditions for the most of PCRs were as follows: initial denaturation at 94ºC for 10 min, followed by 36 cycles of 94ºC for 45 sec, 58ºC for 45 sec, and 72ºC for 10 min, a final extension at 72ºC for 10 min. Some exons amplified in a slightly higher temperature 60ºC or 62ºC. PCR conditions were the same almost for all exons except changing annealing temperature which fluctuated between 56ºC and 60ºC. The purity of the products were verified by gel electrophoresis using 0.8% agarose gel stained with ethidium bromide and images of the gels were visualized by UV transillumination and captured with a digital camera.

3.5. Sanger sequencing

The Sanger sequencing assays were performed by the sequencing platform of the McGill University and Genome Quebec Innovation Centre using forward or reverse sequencing primers designed for PCR reactions which covered a designated exon and splice sites of the exon. The results were analyzed using the Sequencher software program. Identified mutations were analyzed *in silico* by ENSEMBL, BLOSSUM analyses, SIFT and Polyphen to determine their significance.

3.6. Mutation analysis

Homozygous chromosomal segments were analyzed for known retinal detachment and RP genes provided in RetNet (Retinal Information Network, tables of genes and loci causing inherited retinal diseases) gene list (https://sph.uth.edu/retnet/sum-dis.htm#A-genes). Causal genes for the given

disease were sequenced completely to exclude all possible mutations. If a patient had several homozygous regions carrying different known genes for the given disease, the screening of the genes was started from a gene located in the biggest homozygous segment. Primers for amplification of the coding exons and splice junctions were designed using ExonPrimer (http://ihg.gsf.de/ihg/ ExonPrimer.html/ provided in the public domain by the Institute for Human Genetics, Technical University of Munich, Germany) and Primer3 (v. 0.4.0) program (http://frodo.wi.mit.edu/) (Table 18).

CHAPTER 3: RESULTS

1. Overview

First, we phenotyped all consanguineous families diagnosed as NCRNA or arRP from Pakistan with one or more affected siblings. DNA was obtained after informed consent. We performed homozygosity mapping by Illumina 610 K SNP micro-array, a new strategy developed by our laboratory, for all NCRNA and RP consanguineous families by the genotyping platform of the McGill University and Genome Quebec Innovation Centre. Regions of interest (overlap of homozygous regions of siblings and overlap of two families) were further analyzed using the Illumina Genome Viewer and P-link software taking into consideration the criteria for significant homozygous regions (i.e >100 consecutive homozygous SNP markers). Then we performed exome capture by Roche Nimblegen Sequence Capture Arrays for 3 families with more than one affected siblings (RNC2 and RNC3, both have 2 affected siblings, RNC14 has 3 affected siblings) and 1 RP (RP3) family. The rest of families have been screened by RetNet chip (243 retinal dystrophy genes provided in the Retinal Information Network, https://sph.uth.edu/retnet/sum-dis.htm#A-genes) and those patients negative for the RetNet chip (undecided cases) went to WES and NGS (the Illumina HiSeq 2000) at the department of Molecular and Human Genetics located in Human Genome Sequencing Center, Baylor College of Medicine, Houston, Texas, USA. Genes/mutations identified and shared by the siblings and between the families screened using PCR and Sanger sequencing.

Homozygosity mapping of consanguineous patients with recessive diseases is a well-known tool to identify homozygous regions that may contain the causal gene.

Both consanguineous and outbred patients harbor homozygous regions (den Hollander *et. al*. 2007) [74] and the top 5 largest areas often contain the gene of interest. Based on this tool, first, through homozygosity mapping (using SNP micro-arrays) we identified an identity by descent regions (IBD) of chromosomes in all Pakistani families with one or more affected siblings. We performed a large study on consanguineous patients with NCRNA and RP and found that a significant number of consanguineous patients carry significant homozygous regions and the causal gene with mutations lies in the largest or second largest interval. In all, we identified 7 NCRNA and 14 RP families in whom their homozygous regions did not contain any known NCRNA and RP genes or the genes that resided in the regions were negative for pathogenic mutations. These patients moved to next step, the RetNet chip followed by NGS, to identify the deleterious mutant gene.

Genetic studies on the founder population of Pakistan with RP and NCRNA, have led us to some surprising and interesting findings.

2. Results for NCRNA patients

In a preliminary study of our cohort, we prescreened families for known NCRNA and retinal detachment genes resided in one of the top homozygous segments documented by the SNP array. We used P-link software to analyze homozygous regions and direct sequencing of these genes helped us to successfully genotype 8 NCRNA families with candidate genes located in the regions of interest (Table1). Table1 demonstrates the successfully genotyped families with the help of SNP genotyping and the genes that excluded from the study.

Fam. No	Chr.	Kb	Candidate Gene	IBD Rank	Successfully genotyped with Candidate Genes	Excluded Genes
RNC4-5	10	9714	*ATOH7*	Middle	YES	
RNC5-4	10	56048.2	*ATOH7*	Biggest	YES	
RNC6-3	10	31984.6	*ATOH7*	2^{d} biggest	YES	
RNC7-4	X	58365	*NDP*	2^{d} biggest	YES	*ZNF408*
	11		*LRP5*	13th biggest		
	11		*ZNF408*	15th biggest		
RNC8-3	7	1010.11	*TSPAN12*	Last		*TSPAN12*
RNC9-3	7	47265.6	*TSPAN12*	Biggest	YES	
RNC11-3	7	1014.65	*TSPAN12*	Smallest		*TSPAN12*
RNC13-3	7	39214.4	*TSPAN12*	Biggest		*TSPAN12*
RNC14-13			NO ANY			
RNC17-2	7	10360.7	*TSPAN12*	7th biggest	YES	
RNC17-3		25567.7		2^{d} biggest		
RNC19-3	7	17489.4	*TSPAN12*	2^{d} biggest		
RNC19-6		2401.01		9th biggest		
RNC19-3	11	20680.6	*FZD4*	Biggest		*FZD4*
RNC19-4		46448.3				
RNC19-6		19193.3				
RNC19-3	11	13341	*LRP5*	3^{d} biggest	YES	
RNC19-4				2^{d} biggest		
RNC19-6				2^{d} biggest		

Table 1.Candidate genes for NCRNA families in their IBD regions.

Seven families harbored mutated genes in their top IBD (Identity by Decent) regions, the rest of known genes were excluded from the study for the given families.

Homozygous regions of three different consanguineous Pakistani NCRNA families overlapped each other and helped us to develop a hypothesis that causal gene may be located in this interval (Table2). Based on this hypothesis we were able to choose a candidate gene, KIT ligand (*KITLG*) for those families in the interval with the help of Suspect, an online computational tool for prioritizing candidate genes in terms of their sequence homology with *ATOH7* and other retinal developmental genes (http://www.genetics.med.ed.ac.uk/suspects/). *KITLG* plays an essential role in the regulation of cell survival and proliferation, neural cell development, hematopoiesis, stem cell maintenance, gametogenesis, mast cell development, migration and function, and in melanogenesis (http://genome.ucsc.edu). We then tested the gene by Sanger sequencing and excluded harmful mutations.

Amount of Overlapped Families	Chr.	Overlap Region	Candidate Gene
3 families (RNC10, 12, 14)	12	86, 792, 574 - 87, 544, 847	*KITLG*

Table2. IBD overlap region for undecided NCRNA families.

Overlap region for RNC10, 12, 14 families and a candidate gene by SUSPECT online tool.

Thusfar, we have five types of results; a novel and reported mutations in a known gene for NCRNA, the transcription factor *ATOH7*, mutations in the gene (*LRP5*) associated with autosomal dominant and autosomal recessive FEVR, a disease with overlap with NCRNA, novel recessive mutations in the known gene that cause dominant and recessive FEVR disease (*TSPAN12*), novel mutations in

Norrie Disease (*NDP*) gene and finally we have identified NCRNA families that do not seem to be mutated in any known retinal detachment genes.

2.1.Known genes for NCRNA, *ATOH7 (*Atonal homolog 7)

Our study shows that *ATOH7* is not the only gene to cause NCRNA in children and we also show that the disease spectrum is much wider than previously established. We have 21 Pakistani families with NCRNA phenotype and only 3 families had ATOH7 in the largest IBD regions (our mutation analysis confirmed this). What was exciting is that two of these families harbored reported mutation in the only known gene for NCRNA which is common in a founder population of North Khorasan, Iran [5] and seven out of our 21 NCRNA families did not harbor neither *ATOH7 n*or any other retinal detachment gene in any of their homozygous regions, suggesting that we have many families with new genes for NCRNA. However, we screened all our patients for *ATOH7* exonic and promoter mutations. All three families (RNC4, RNC5 and RNC6) were checked for exonic and promoter mutations. 2 families (RNC5 and RNC6) had reported 6, 523 bp deletion that located 20 kb upstream from *ATOH7* gene (phenotypes of the patients are shown in Figure9 and 10) which is common in North Khorasan, Iran, among descendants of a Kurdish founder population[1]. Segregation analyses done for these families conducted as described by Ghiasvand *et. al.* (2011) [5] (see Figure 12). We have found a novel homozygous missense mutation p.Arg42Pro, altering an arginine for a proline residue in *ATOH7* in the third family. PolyPhen, Polymorphism Phenotyping tool, which predicts possible impact of an amino acid substitution on the structure and function of a human protein shows that this

mutation is probably damaging (Table4) and conservation analysis by UCSC genome browser shows the significance of the residue (Figure9).The list of identified mutations is shown in Table3.

Family	Chr.	Gene	IBD rank	Change	Reported Mutation	Co-Segregation
RNC4	10	*ATOH7*	Middle	c.G125C p.R42P hmz.	new	YES
RNC5	10	*ATOH7*	Biggest	6, 523bp Promoter deletion hmz.	YES	YES
RNC6	10	*ATOH7*	2^{d} biggest	6, 523 bp Promoter deletion hmz.	YES	YES

Table 3.*ATOH7* gene mutations identified by SNP genotyping method.

Gene and Mutation	Polyphen2	SIFT	Blosum62
*ATOH7,*c.G125C, p.R42P	Probably damaging (score 0.999)	Affect protein function (score of 0.00)	Score -2

Table 4.Summary of analysis done by bioinformatic tools for *ATOH7* gene mutations.

Summary of analysis done by bioinformatic tools to predict the pathogenic nature of the mutations in *ATOH7* gene; Blosum62 scores range from +3 to -3, negative scores are presumed to be damaging.

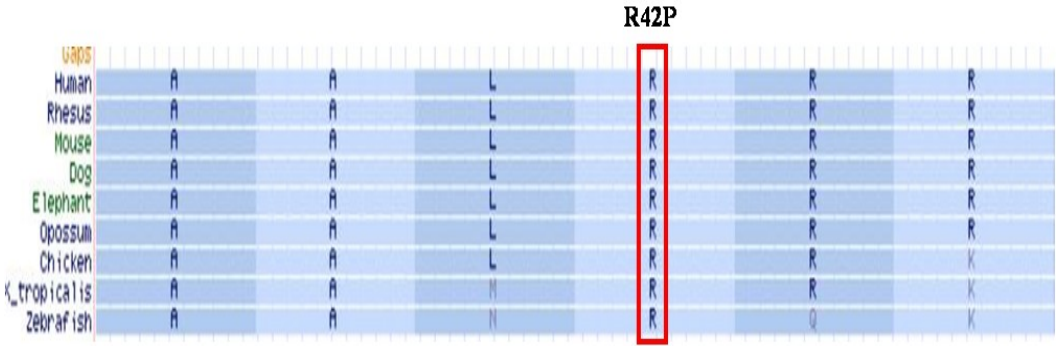


Figure 9. Mutation conservation analysis by UCSC Genome Browser.

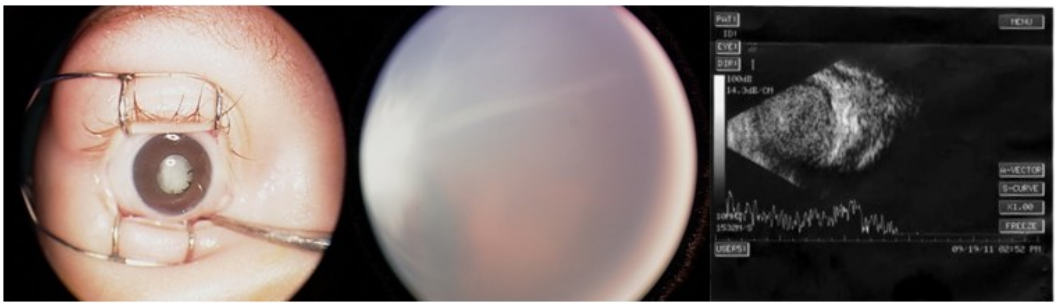


Figure10. RNC5-4 retinal photographs and B scan result.

White pupil, retinal fold and disorganized B scan caused by the variation in *ATOH7* gene.

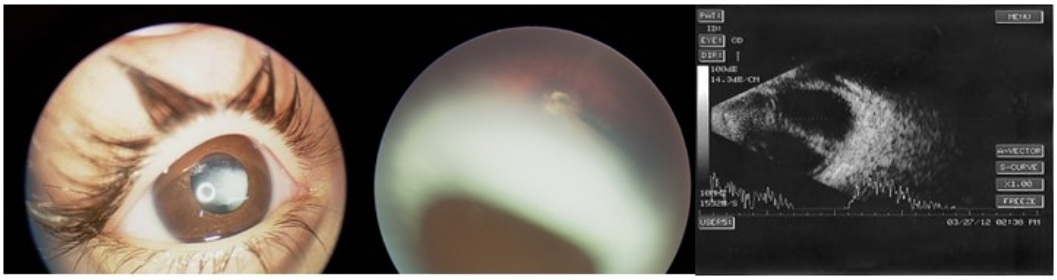


Figure11. RNC6-3 retinal photographs and B scan.

The white pupil, the funnel shaped RD, retinal fold and completely disorganized B scan show the severity of the disease in the patient.

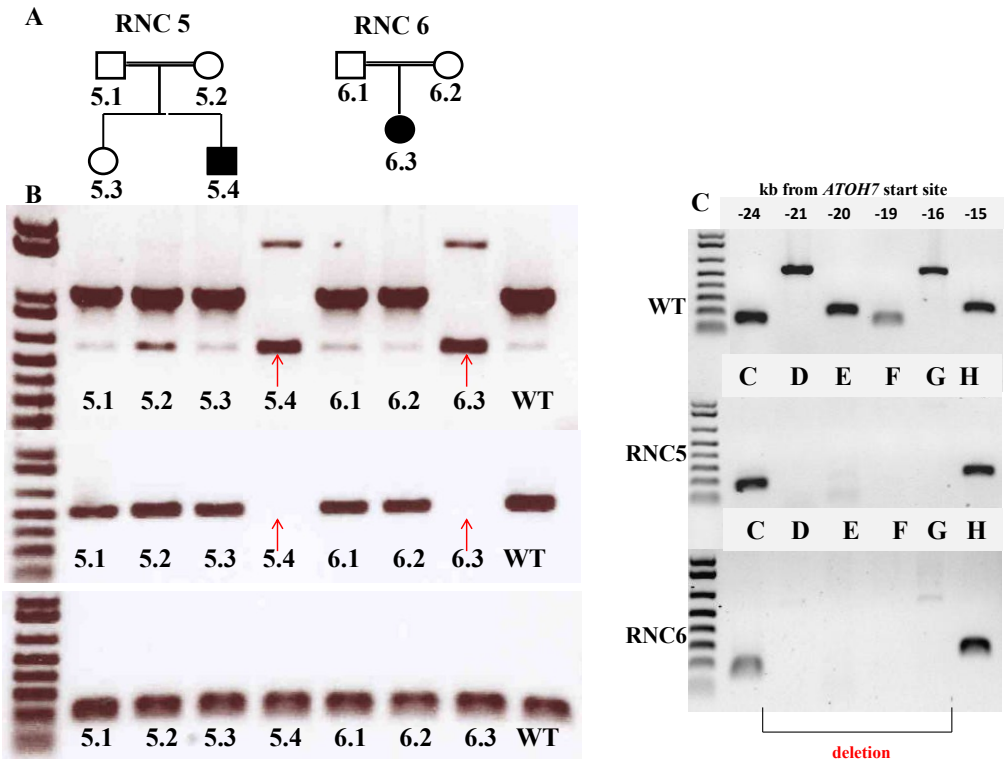


Figure12. Homozygous 6523kb deletion in 5'*ATOH7* in RNC5 and RNC6 families.

A. RNC5 and RNC6 family pedigrees, B. Co-segregation results for the families; arrows show deletion in affected family members compared to wild type (WT) control bands, C. PCR based deletion confirmation in the families (Gisvand *et. al.* (2011)[5].

2.2.Mutations in *LRP5* (low density lipoprotein receptor-related protein)

We found mutations p.Gly610Arg, p.Asp434Asn, p.Trp79Arg and p.Gly1401Asp in *LRP5*, the gene that encodes a transmembrane low-density lipoprotein receptor (Table5) and plays a key role in eye development and in skeletal homeostasis and many bone density related diseases [75]. The altered residues are evolutionarily conserved (Figure 13 and 14). The eye phenotype consisted of shallow anterior

chamber (AC) due to anteriorly displaced lens and iris, posterior synechia, retrolenticular fibrovascular plaque, corneal opacity, leukocoria, delayed growth, retinal fold (in some patients), complete retinal detachment and skeletal abnormalities in some patients (Figure15 and 16). Our whole genome SNP genotyping for the 2 families identified significant IBD region overlap between the RNC2 and RNC3 families at chr.11 (7.8Mb, containing 17 genes). Although *LRP5* gene is known for mild retinal detachment and was located in this region, the patients' phenotype was so severe that we could not diagnose the patients as FEVR. To confirm the diagnosis properly we sent these two patients for NGS. NGS results indicated that homozygous amino acid changes in *LRP5* were causal for those 2 families (splice site or frame shift mutations in the homozygous regions) as a result of FEVR. Further SNP array analyses showed that RNC7 (a novel mutation, Table6 shows summary of analysis done by bioinformatic tools to predict nature of the mutation) and RNC19 families have mutations in the same gene also. The mutations perfectly co-segregated in the families.

As p.G1401D mutation in *LRP5* gene is reported to cause osteoporosis-pseudoglioma [75] we checked bone mineral density (BMD) in RNC19-4 patient and the result confirmed that the patient has reduced BMD and osteoporosis. The rest of families were not able to come back for the given test.

Changes in *LRP5* gene sequences have been found in additional three families (RNC12, RNC16 and RNC20) by RetNet chip analysis which should be validated with Sanger sequencing and checked for segregation within the families. Pedigrees of the successfully genotyped families are shown in Figure 17.

Family	Chr.	Gene	IBD rank	Change	Reported Mutation	Co-Segregation
RNC2	11	*LRP5*	2^{d} biggest	c.G1828C p. G610R	YES	YES
RNC3	11	*LRP5*	4th biggest	c.G1300A p.D434N	YES	YES
RNC7	11	*LRP5*	Middle	c.T235C p.W79R	NEW	YES
RNC19	11	*LRP5*	2^{d} biggest	c.G4202A p.G1401D	YES	YES

Table 5. Homozygous *LRP5* mutations identified by SNP genotyping and NGS methods.

Gene and Mutation	Polyphen2	SIFT	Blosum62
LRP5,c.T235C p.W79R	Probably damaging (score 1.000)	Affect protein function (score of 0.00)	Score -3

Table 6.Summary of analysis done by bioinformatic tools for *LRP5* gene variations.

Analysis done by bioinformatic tools predicted the nature of the mutation in *LRP5* gene as pathogenic; Blosum62 scores range from +3 to -3, negative scores are presumed to be damaging.

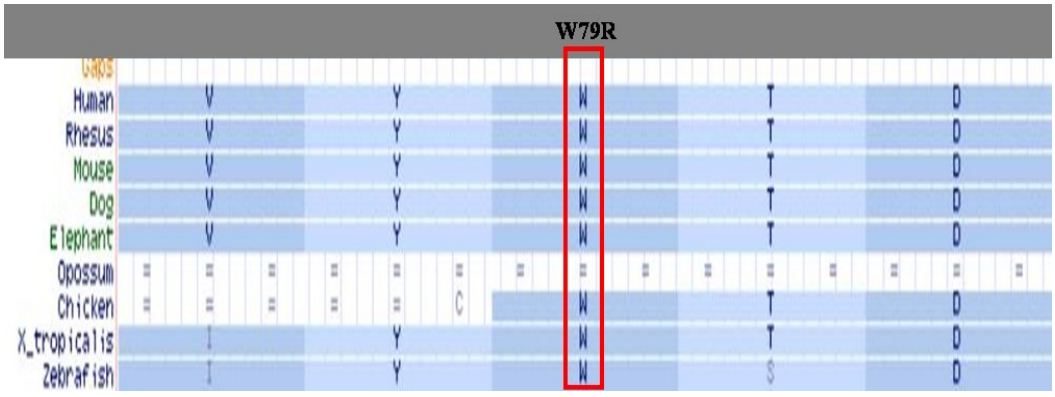


Figure 13. Mutation conservation analysis for *LRP5* by UCSC Genome Browser.

Mutations:	G610R	D434N	G1401D
Human LRP5	ADRNGGCSH	LYWTDTGTD	LFVMGGVYF
Mouse LRP5	ADGNGGCSH	LYWTDTGTD	LFVMGGVYF
Rat LRP5	ADGNGGCSH	LYWTDTGTD	LFVMGGVYF
Xenopus LRP5	ADNNGGCSH	LYWTDTGTD	VFVMGGLYF
Human LRP6	AEENGGCSH	LYWTDTGTD	IFVSGTVYF
Mouse LRP6	AEDNGGCSH	LYWTDTGTD	IFVSGTIYF
Drosophila arrow	AVRNGGCSH	LYWTDTVTD	ITIFSIVYL

Figure 14.Disease-associated mutations in *LRP5* gene.

The altered amino acid residues are shaded; the altered residues are evolutionarily conserved in the given species. (from Minrong Ai *et. al.* 2005) [75].

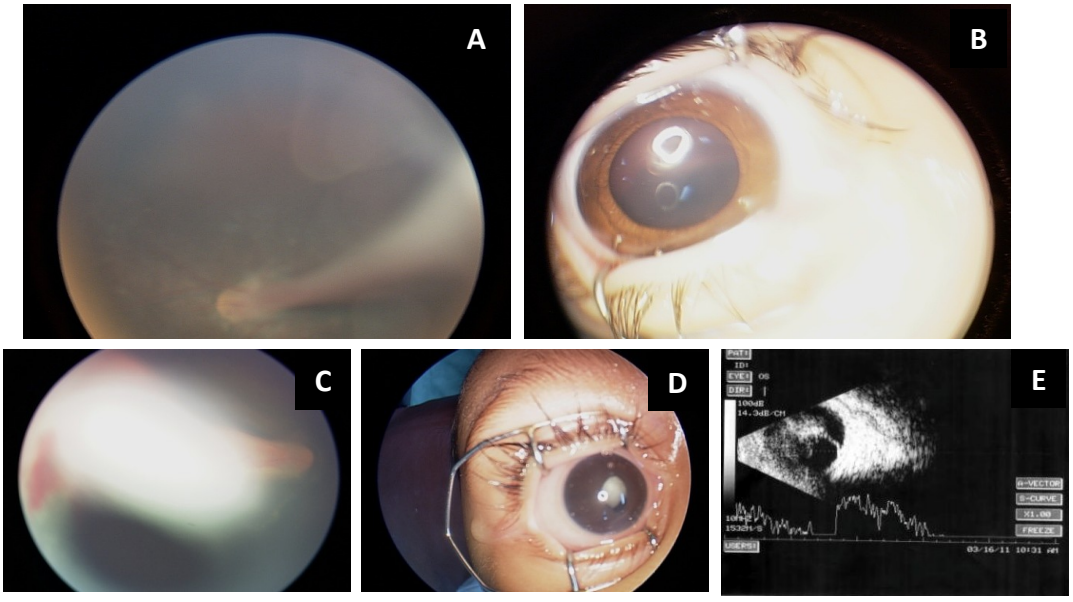


Figure 15. RNC2-5 and RNC7-4 retinal photographs.

RNC2-5 (A and B) and RNC7-4 (C, D and F) retinal photographs. A and B show retinal fold and white pupil, C, D and F show retinal fold, white pupil and completely destroyed B scan subsequently.

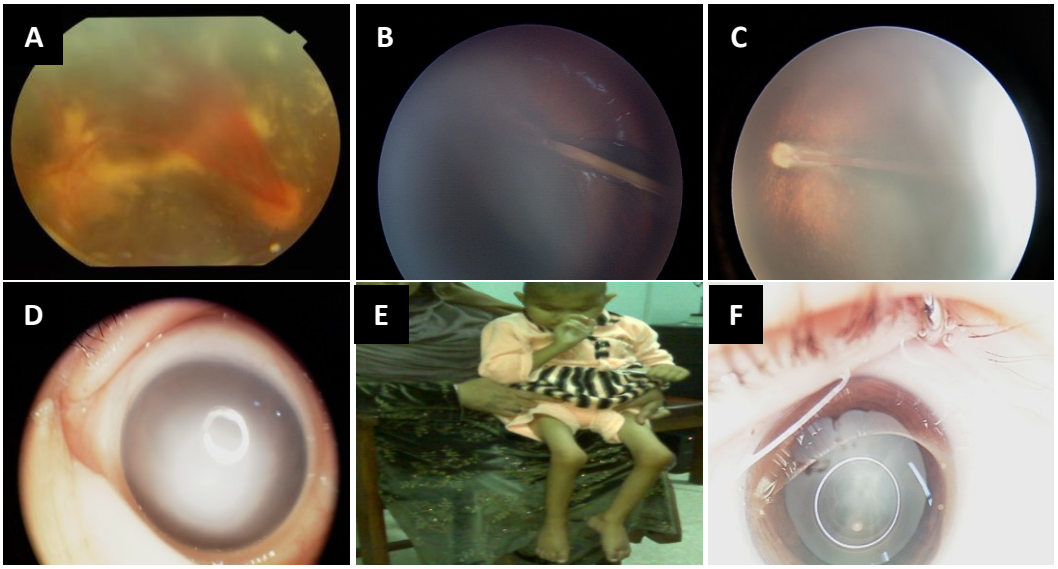


Figure 16.Retinal photographs for RNC3 and RNC19 family members.

A and B are retinal photographs for RNC3 and C, D, E and F for RNC19 family members; A and B show severe retinal fold caused by p.G610R mutation in *LRP5* gene in RNC3-4 and RNC3-7 probands subsequently. C, D, E and F belong to RNC19 family members with p. G1401D mutation and show retinal fold, leucokoria and skeletal abnormalities (RNC19-3, RNC19-4 and RNC19-6 subsequently).

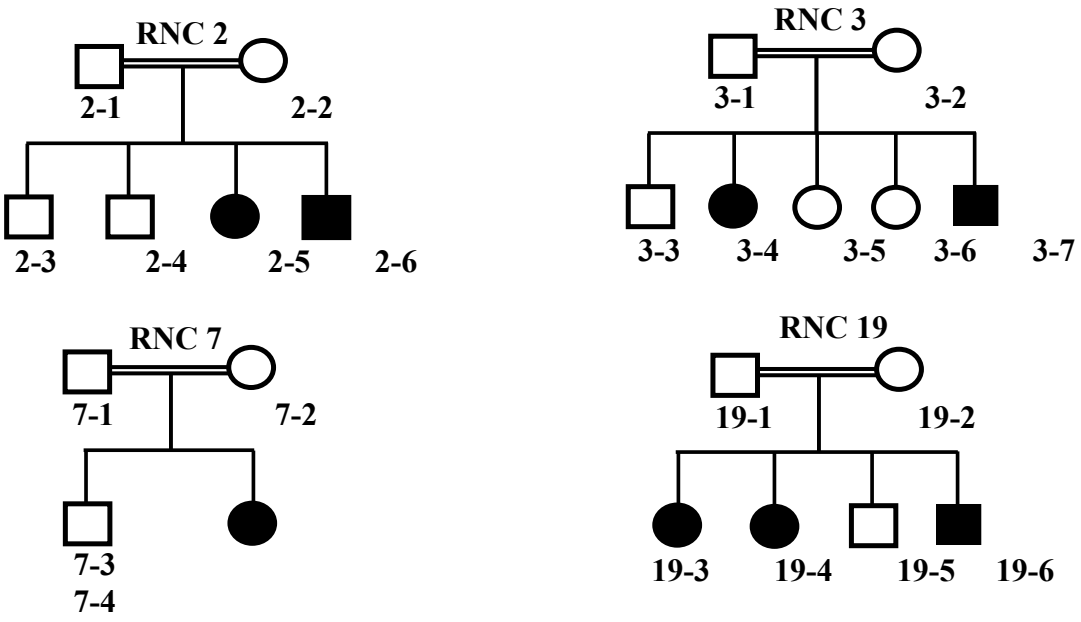


Figure 17. Successfully genotyped RNC2, RNC3, RNC7 and RNC19 family pedigrees positive for homozygous *LRP5* mutations.

2.3.Mutations in *TSPAN12* (Transmembrane 4 superfamily member 12)

We found for the first time, recessive mutations IVS4-2A>G (hmz) and p. Pro141fsX21 (hmz) in *TSPAN12* (Table7), the gene that encodes cell-surface proteins that are characterized by the presence of four hydrophobic domains and mediate signal transduction events that play a role in the regulation of cell development, activation, growth and motility [76]. We revisited the phenotypes and found leukocoria, corneal opacity, shallow AC, posterior synechia and total retinal detachment (Figure19). Six families had *TSPAN12* as a candidate gene in their significant homozygous regions. *TSPAN12* is currently known to cause adFEVR. It is not known to cause ar disease and severe retinal detachments. Sanger sequencing results showed that RNC9 and RNC17 families carry splice

site and frame shift mutations in *TSPAN12* gene respectively while the other families did not show any harmful mutations in the given gene. Berkeley Drosophila Genome Project (BDGP), splice site predictor analysis tool, showed that IVS4-2 A>G mutation in RNC9 deletes the acceptor site (Figure18).

Family	Chr.	Gene	IBD rank	Change	Reported Mutation	Co-Segregation
RNC9	7	*TSPAN12*	Biggest	IVS4-2 A>G hmz.	NEW	YES
RNC17	7	*TSPAN12*	2^{d} biggest	c.423del T p. P141fsX21 hmz.	NEW	YES

Table 7.*TSPAN12* mutations in successfully genotyped NCRNA families.

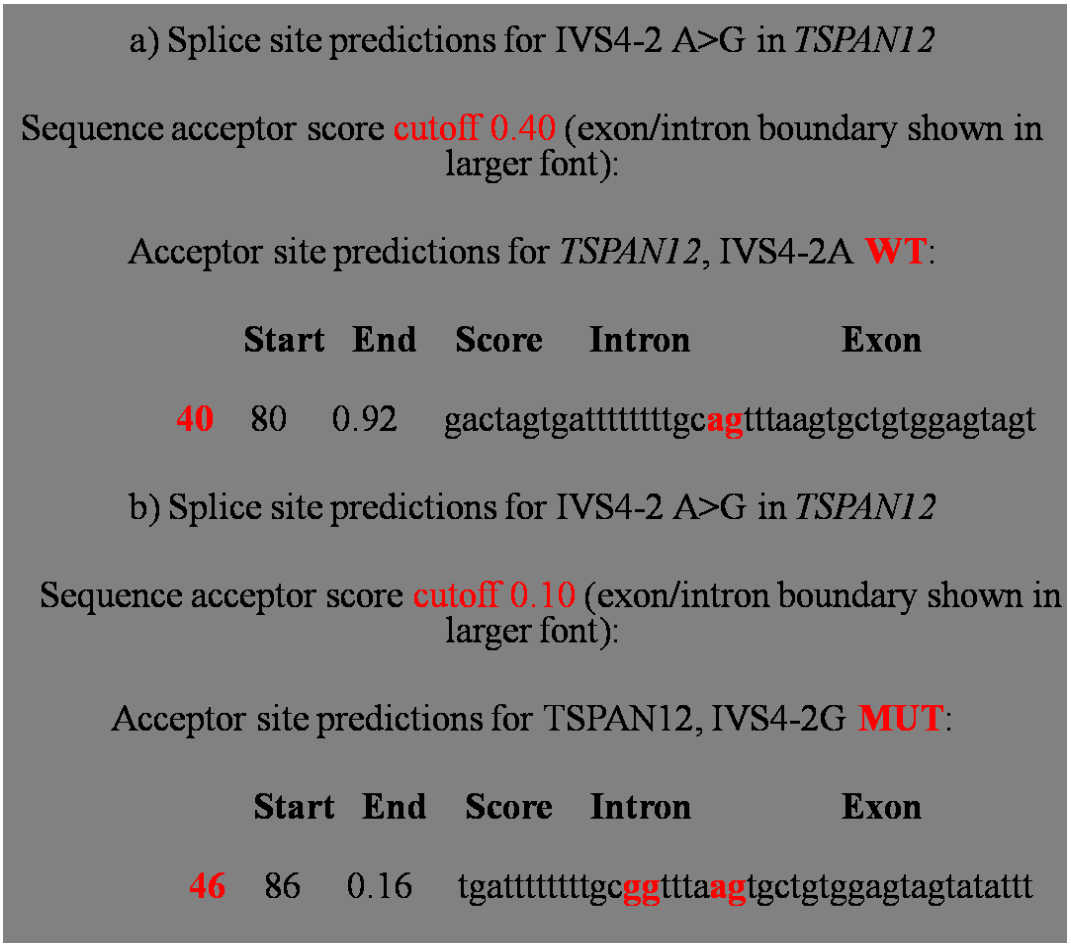
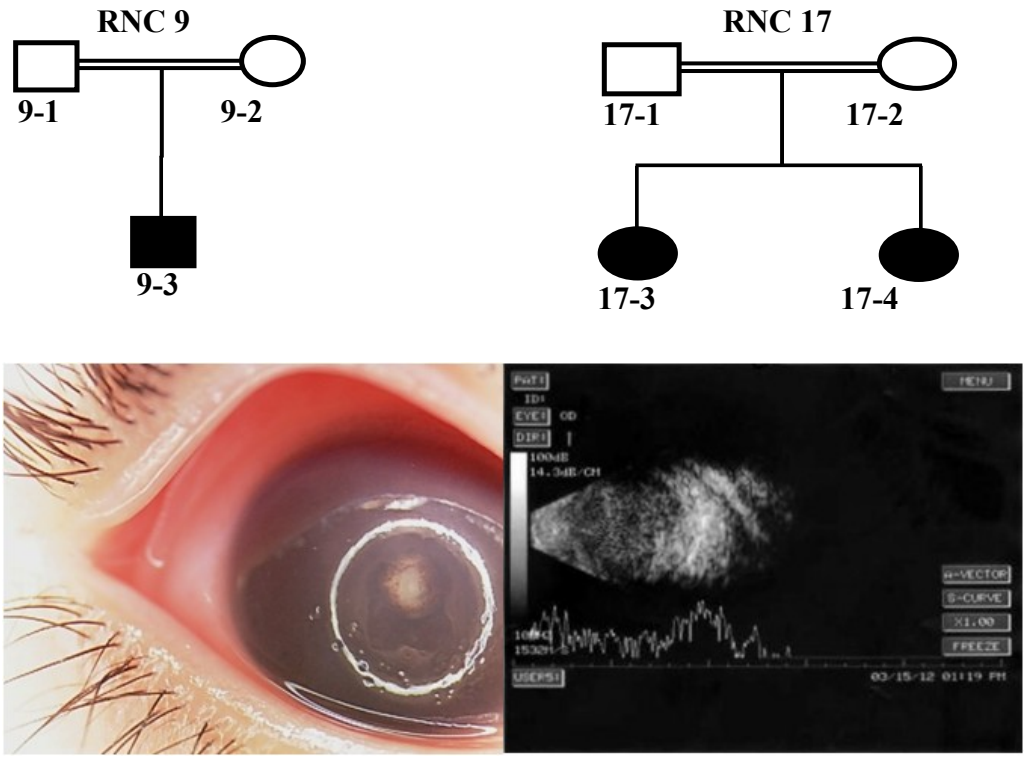


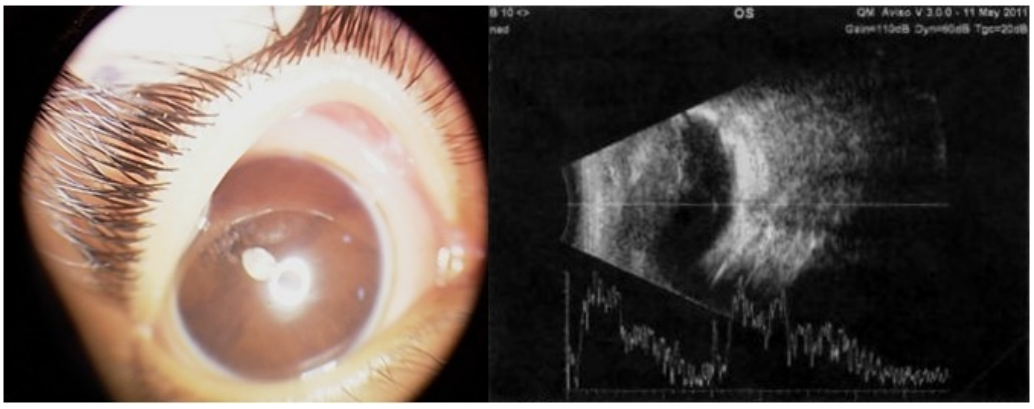
Figure18. Splice site mutation analysis for *TSPAN12*.

Berkeley Drosophila Genome Project (BDGP), splice site predictor analysis tool, showed that IVS4-2 A>G mutation in RNC9 deletes the acceptor site. First result corresponds to wild type sequence with a score of 0.92, the second result corresponds to mutant sequence with very low splice site score 0.16

Co-segregation analysis showed that mutations are perfectly segregates in the families. The other 3 families carrying *TSPAN12* gene in their IBD regions were excluded for harmful mutations.



RNC9-3 *TSPAN12* IVS4-2A>G



RNC17-3 *TSPAN12* p. P141fsX21

Figure 19. *TSPAN12* mutation phenotypes in successfully genotyped families.

White pupils and destroyed B scan confirms severity of the disease and complete retinal detachment.

2.4. Mutations in Norrie disease gene (*NDP*)

With the help of RetNet chip and NGS we found mutations in *NDP*, the gene that encodes a protein that plays a key role in retinal vascularisation and activates the Wnt signaling pathway with the help of its co-receptors, *FZD4* and *LRP5*. The gene also is thought to be implicated with neural cell differentiation and proliferation, also neuroectodermal cell-cell interaction (http://www.genecards.org/cgi-bin/carddisp.pl?gene=NDP). Mutation in this gene cause X-linked exudative vitreoretinopathy.The patients' phenotype consistent with the phenotype of previously described families (Ghiasvand *et. al.* 2011) [5] and with the families shown above with the mutations in *ATOH7, LRP5* and *TSPAN12* genes; bilateral corneal opacities, shallow AC, posterior synechia, retrolenticular fibrovascular plaque and complete retinal detachment. A novel homozygous 244 bp deletion starting from the end of the 3^{d} exon deleting 67bp exon and 177bp intron (ending position is 402) in *NDP* in RNC14 family was identified by NGS performed in the sequencing platform of the McGill University and Genome Quebec Innovation Centre and a novel nonsynonymous p.Cys69Gly mutation in RNC11 family with the help of NGS conducted in Human Genome Sequencing Center, Baylor College of Medicine, Houston, Texas, USA (Table8). A novel nonsynonymous p.C69G mutation in RNC11 family is predicted to be probably damaging with a score of 0.997 with the help of PolyPhen (Table9) and UCSC Genome Browser shows that the residue is evolutionally conserved (Figure20). Mutations segregate within families (Figure21).

Family No	Chr.	Gene	Mutation	Reported Mutation	Co-Segregation
RNC11	X	*NDP*	c.T205G p.C69G	NEW	YES
RNC14	X	*NDP*	c.336_402IVS4del	NEW	YES

Table 8. RNC families successfully genotyped by NGS.

Gene and Mutation	Polyphen2	SIFT	Blosum62
*NDP*c.T205G p.C69G	Probably damaging (score 0.997)	Affect protein function* (score of 0.00)	Score -3

Table9. Summary of analysis done by bioinformatic tools for *NDP* gene variations.

The summary of analysis done by bioinformatic tools predicted the pathogenic nature of the mutation in *NDP* gene; Blosum62 scores range from +3 to -3, negative scores are presumed to be damaging.*(WARNING!! This substitution may have been predicted to affect function just because the sequences used were not diverse enough. **There is LOW CONFIDENCE in this prediction**).

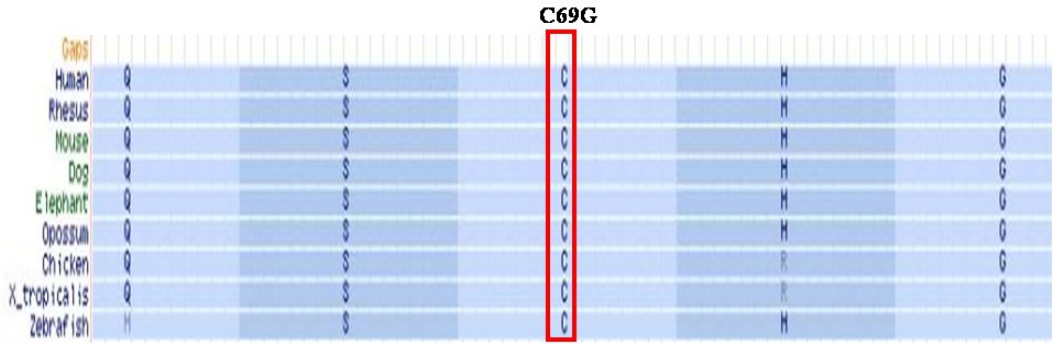


Figure20. Mutation conservation for *NDP* variation by UCSC Genome Browser.

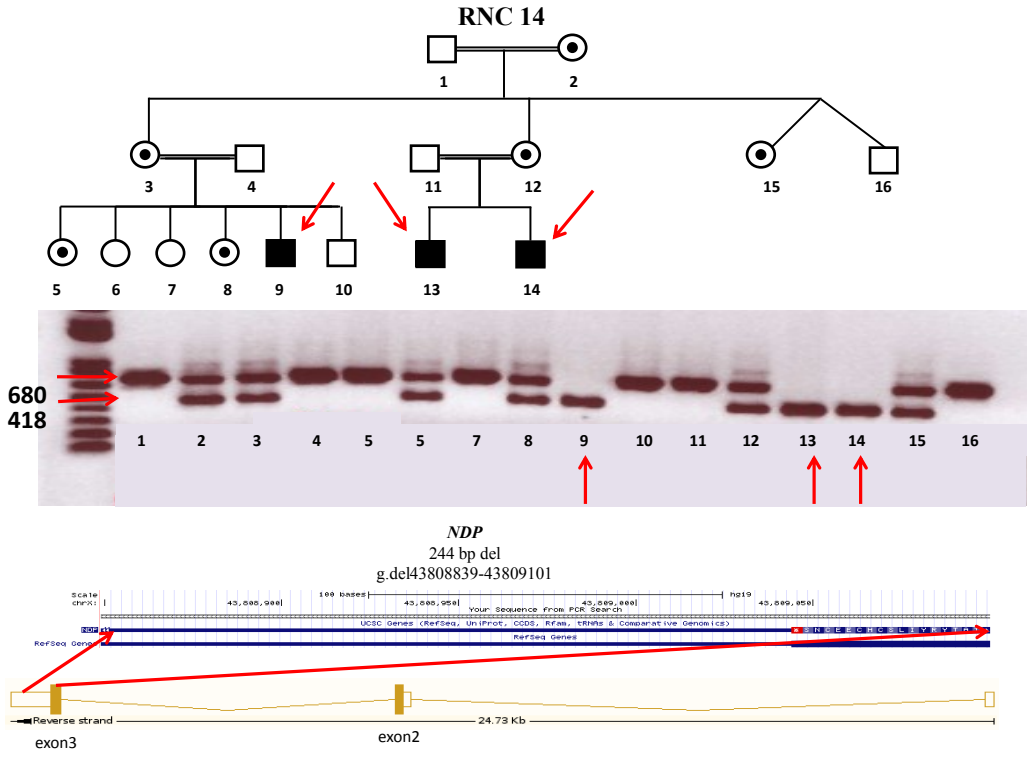


Figure21. RNC14 family pedigree and co-segregation result for *NDP* gene deletion.

Arrows show the affected members of the family and family members with double bands (correspond to the sizes 418 and 680) are heterozygous carrier females.

2.5. Mutations in new genes

Now we have NGS results for 7 families suggesting that these families have mutations in new genes involved in NCRNA. The revisited phenotype consists of leukocoria, corneal opacity, disorganized B scan. In these seven families we suggest the following candidate genes based on NGS preliminary filtration result (Table 10). These genes are expressed in retina and involved in different types of retinal diseases and may be involved in NCRNA also. These families are still in investigation for candidate genes. *LRP5* is a known gene, so say 4 genes are novel candidates, possibly new genes for NCRNA;

Alstrom syndrome 1 (*ALMS1)* gene; the encoded protein is required for the normal formation and/or maintenance of primary cilia and involved in PCM1-dependent intracellular transport [83]. Variants in this gene are responsible for ar disease called Alstrom syndrome [83] which characterized by cone-rod retinal dystrophy leading to blindness, hearing loss, childhood obesity and type 2 diabetes mellitus [MIM:203800].

Cadherin-related family member 1 (*CDHR1*) gene; the encoded protein is thought to be involved in the formation and maintenance of neuronal networks [76]. Mutations in *CDHR1* are reported to cause cone-rod dystrophy type 15 (CORD15) [MIM: 613660] [84].

Centrosomal protein 164kDa (*CEP164*) gene; protein encoded by this gene plays role in microtubule organization, DNA damage response, and chromosome segregation. The protein is required for assembly of the primary cilium. Variants in this gene lead to nephronophthisis-related ciliopathies [76].

These genes will be studied for potential involvement in NCRNA.

Moreover, we have identified several chromosomal regions that overlap between families that still under investigation for the causal genes (Table11). These overlap regions may help us to narrow down the region that carries the disease gene. These regions will be studied for candidate genes also.

Family	Candidate Genes	Mutation Type	Change
RNC12-3	*LRP5*	nonsynonymous	c.C2254T:p.R752W
	ALMS1	nonsynonymous	c.A5242G:p.T1748A
		nonsynonymous	c.C3263G:p.T1088R
RNC15-2	*CDHR1*	nonsynonymous	c.G1471A:p.V491M
		nonsynonymous	c.C2473A:p.P825T
RNC 16-3	*LRP5*	nonsynonymous	c.G425A:p.R142Q
RNC 18-3	*CEP164*	nonsynonymous	c.C1484G:p.P495R
RNC 20-3	*LRP5*	nonsynonymous	c.T1622C:p.L541P
RNC 21-6	*CDH23*	nonsynonymous	c.C4892T:p.A1631V
		nonsynonymous	c.G5026A:p.A1676T

Table 10.Candidate genes for undecided NCRNA families.

Number of Overlapped Families	Chr.	Overlap Regions
2 (RNC10, 12)	3	49, 660, 595-49, 854, 206
2 (RNC1, 10)	6	27, 394, 768- 27, 972, 886
2 (RNC10, 12)	7	91, 118, 405- 92, 171, 343
2 (RNC10, 12)	12	86, 792, 574 – 87, 544, 847
2 (RNC1, 12)	17	55, 438, 497- 56, 587, 146

Table 11. Undecided NCRNA families with overlap IBD regions.

3. DISCUSSION

In this study, 28 Pakistani blind children from 17 consanguineous and 4 non consanguineous families with the diagnosis of NCRNA were analyzed with variety of gene-identifying techniques, including SNP microarrays searching for homozygous regions. All the patients were from Northern Pakistan where consanguineous marriages are very prevalent. As expected, the majority of the patients in our cohort carried significant homozygous regions in their genomes as a result of either consanguineous marriages or consanguineous union existing in the region.

In 11 (65%) of 17 consanguineous families, homozygosity mapping successfully identified the region and subsequently a disease-causing mutation in the only known NCRNA gene or other retinal disease genes. It is important to note that, the disease gene was in the largest or second largest homozygous region in 5 of the 11 successfully genotyped families. In accordance with our previous study [74] the data obtained in this current study suggests that the ranking of the IBD regions can be very helpful to identify which homozygous segment contains the possible underlying causal and mutant gene. In the rest of families the known NCRNA and retinal disease genes located in significant homozygous regions were excluded from the study.

Although the clinical diagnosis and strict entry criteria of all children was NCRNA, in eight families, the molecular diagnosis determined that the disease process was in fact a new and severe form of FEVR. FEVR is a rare disease with a wide severity spectrum of retinal disease ranging from essentially normal to

avascular peripheral retina to exudative retinal detachment. It is not known to be associated with the severe developmental and congenital retinal detachment in babies and children. Therefore, we have expanded the phenotypic spectrum of FEVR, a severe retinal detachment phenotype that clinically overlaps with NCRNA. In addition, we identified the identical large deletion (6, 523 bp del) in *ATOH7* gene found in the Kurdish founder population of Northern Iranian with a high incidence of NCRNA (Ghiasvand *et. al.*, 2011), suggesting genetic overlap between the Iranian and Pakistani populations and a founder mutation in *ATOH7* gene links the two populations.

Our molecular genetic study of clinically evaluated and diagnosed patients with NCRNA has revealed five important patterns. First, our work illustrates that NCRNA and FEVR overlap clinically; FEVR can be so severe that it mimics NCRNA; we show for the first time that the NCRNA phenotype can be caused by FEVR mutations. Second, we found the *ATOH7* deletion links the Pakistani population to the Iranian population. Possible explanations for this could be a common ancestor for both populations or substantial inter breeding aroused from the patients' ancestors who all lived in the same general large geographical area. Third, we have found novel mutations, hmz c.G125C, p.R42P mutation in *ATOH7*, hmz c.T235C, p.W79R mutation in *LRP5*, hmz splice site IVS4-2 A>G and hmz framshift c.423del T, p. P141fsX21mutations in *TSPAN12*, and hmz c.T205G, p.C69G and 244 bp deletion in *NDP* genes. These families were informed about the results and were genetic counseled about family planning and future treatment options. Fourth, we confirmed that recessive mutations in *TSPAN12* cause more severe FEVR phenotype than the previously known

dominant mutations in adFEVR and *TSPAN12* mutations. We identified this discovery independently, but colleagues in Leeds (also working on NCRNA in Pakistani families) identified this and published it first (Poulter *et al*. 2012) [38]. And fifth, and perhaps most exciting is that we have undecided NCRNA families that must have new genes.

In addition, we have used in this study our newly developed RetNet chip which for the first time contains all the currently known retinal disease genes by targeted NGS which allowed us to screen all the genes listed in Retinal Information Network website and create candidate retinal disease gene list for future study, in case if they are involved in new unknown mechanisms.

4. Results for RP patients

4.1.Mutations in known RP genes

We also recruited 16 consanguineous Pakistani families diagnosed with arRP and phenotyped their retinal disease in detail. DNA was obtained from all members, including parents, affected and unaffected siblings. To test the hypothesis that affected siblings with overlapping homozygous (hmz) regions contain the causal mutant gene, we subjected all probands and one other affected sibling from each family to SNP genotyping by Illumina 10 K chip and analyzed the data with P-link software, using our published methods and criteria for significant homozygous regions (i.e >100 consecutive homozygous SNP markers). Well known RP genes (Table12) residing in the overlapping regions was Sanger sequenced for the families and we have determined the causal known genes for 2 families.

Patient No	Candidate genes in IBD	Excluded genes	Successfully genotyped families with candidate genes
RP1	*RPE65*	screened	
	KLHL7	screened	
	CERKL		**Successfully genotyped**
RP2	*CNGA3*	screened	
	USH2A	screened	
	GUCY2D		
	AIPL1	screened	
	GNTPG	screened	
RP3	*RDH5*	screened	
	CABP4	screened	
	BEST1	screened	
	OAT		
RP4	*TULP1*		
	AIPL1		**Successfully genotyped**
RP5	*EYS*	screened	
	CDH23		
	TULP1	screened	
RPC6	*RPE65*	screened	
	NRL	screened	
	RPGRIP1	screened	
	OAT		

Table 12. Candidate genes in RP patients' IBD regions.

Our whole genome SNP genotyping for the families helped us to rule out two RP families, RP1 and RP3, with known mutations in *CERKL* (ceramide kinase-like) and *AIPL1* (aryl hydrocarbon receptor interacting protein-like 1) genes (Table13). Fundus photographs of the families are shown in Figure23 and 24.The found mutations perfectly co-segregated in the families (Figure25).

Family	Chr.	Gene	IBD rank	Change	Reported mutation	Co-Segregation
RP1	2	*CERKL*	Middle	c.C847T p.R283X hmz.	YES	YES
RP4	17	*AIPL1*	Middle	c.G814A p.W278X hmz.	YES	YES

Table 13. Successfully genotyped RP families and their mutations.

The mutation identified in *AIPL1* gene is known to cause LCA in children and originally reported in Pakistani families (Sohocki *et. al*., 2000 [77]). The author also concluded that mutations in this gene accounts for approximately 20% of LCA patients. Our analysis confirmed that affected members of RP4 family are in fact LCA patients and demonstrated that molecular genetic diagnosis is more powerful than clinical diagnosis. Pedigrees of the families are shown in Figure 22.

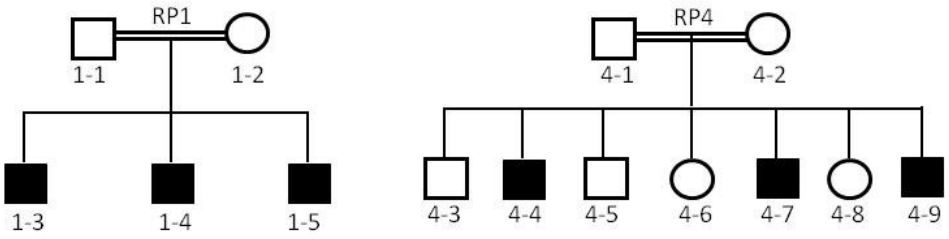


Figure 22.RP1 and RP4 family pedigrees.

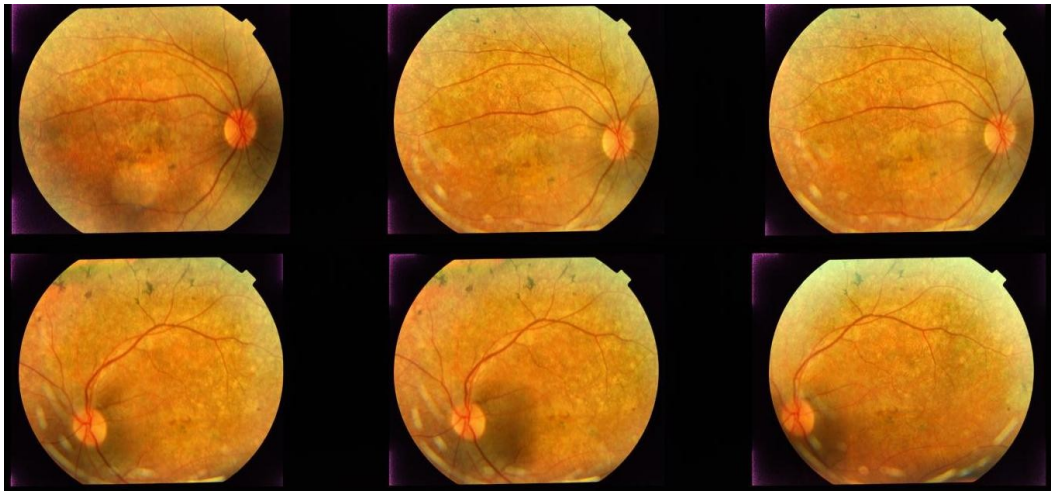


Figure 23. Fundus photographs of Pakistani arRP patient (RP1-3).

RP caused by the mutation in *CERKL*- Ceramide kinase-like isoform b gene.

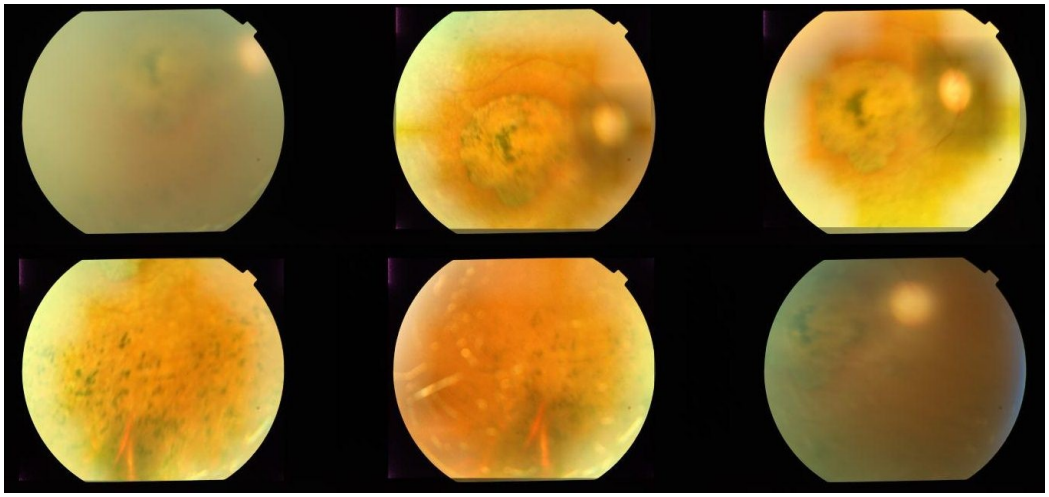


Figure 24. Fundus photographs of Pakistani arRP patient (RP4-4).

RP caused by the mutation in *AIPL1*-Aryl hydrocarbon receptor interacting gene.

Mutation: p.W278X/W278X**,** c.814G>A/814G>A

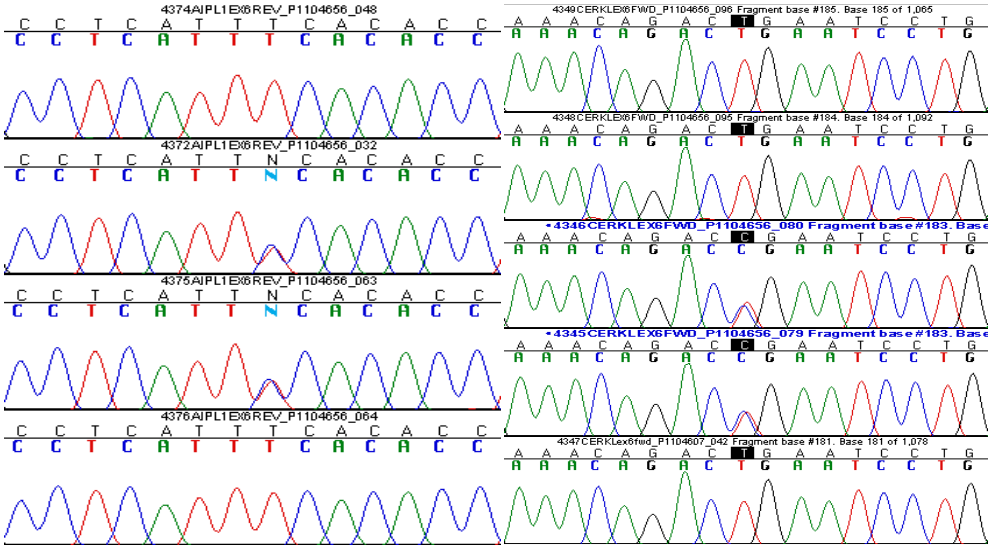


Figure 25. Sanger sequencing results for RP1 and RP4 families.

Stop mutations in *CERKL* (p.R283X/R283X, c.847C>T/847C>T) and *AIPL1* (p.W278X/W278X, c.814G>A/814G>A) co-segregate perfectly causing the disease phenotype subsequently in RP1 and RP4 families.

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4.2. A potential new candidate RP gene

While in several families we are still searching for novel genes and ruling out known ones, in family RP3, we have ruled out all known genes (Table12). First, by SNP genotyping we identified five large hmz regions, ranging in size from 7-23 Mb. One of these hmz regions very likely harbors the causal RP gene, and they reside on chromosome (chr.) 3, 6, 9, 12, and 18. We found known RP and retinal dystrophy (RD) genes and Sanger sequenced *RDH5*, *CABP4* and *BEST1* and excluded harmful mutations. Based on these exclusions, we subjected the proband (RP3) to whole exome capture followed by next generation sequencing (pedigree of the family is shown in Figure26). After preliminary filtering, we have found promising mutations in the proband in six new genes, and we tested each of these

new genes for confirmation. We thusfar found homozygous, potentially damaging mutations in the following six excellent candidates (Table14), but we are not done with our complete filtering. We have identified *BCAR1* (which encodes breast cancer anti-estrogen resistance 1, involved in various cellular events, including migration, survival, transformation, and invasion), *COL4A2* (which encodes a collagen protein), *FAM111B* (which encodes a ubiquitination factor), *SIRPA* (which encodes a member of the signal-regulatory-protein family, and are receptor-type transmembrane glycoproteins known to be involved in the negative regulation of receptor tyrosine kinase-coupled signaling processes), *OVOL1* (which encodes a putative zinc finger containing transcription factor is thought to be involved in spermatogenesis) and *RHOD* (which encodes Ras homolog protein expressed in retina, interacts with protein kinases, binds to GTP and is involved in endosome dynamics) as excellent candidates. Powerful was that some of these candidates had very good variants (from NGS) and were located in the homozygous regions determined by SNP mapping. Systematic NGS data analysis, Sanger sequencing validation, and segregation analysis has been done to identify the pathogenic causal mutations underlying the disease and we have ruled out *BCAR*, *COL4A2*, *OVOL1* and *SIRPA* as they did not segregate in the family. *FAM111B* and *RHOD* both were very good candidate and segregated in the family. To confirm *FAM111B* mutation as a potential disease causing variant we tested *FAM111B* in 40 French-Canadian normal controls and the mutation was found in 3/40 normal controls which lead us to exclude the gene as potential variant. Thusfar *RHOD* is left as a very powerful candidate gene for the family but we are not done with our complete work. The variant found in *RHOD* will

also be analyzed by *in silico* analyses to probe pathogenicity. We will then analyze the mutation in a cohort of 100 normal control Pakistani individuals to test the mutation frequency and we will re-visit the disease phenotype.

Total reads	Chr.	Gene	Exon	Mutation	Mutation type
10	16	*BCAR1*	6	c.G1610A:p.R537H	hmz, nonsynonymous
8	13	*COL4A2*	25	c.A1885G:p.K629E	hmz, nonsynonymous
103	11	*OVOL1*	2	c.G157A:p.E53K	hmz, nonsynonymous
6	20	*SIRPA*	2	c.T131C:p.L44S	hmz, nonsynonymous
12	11	*RHOD*	2	c.C157T:p.R53W	hmz, nonsynonymous
99	11	*FAM111B*	4	c.392delT:p.V131fs	hmz, frameshift deletion

Table14. Homozygous, potentially damaging mutations found in preliminary NGS filtration.

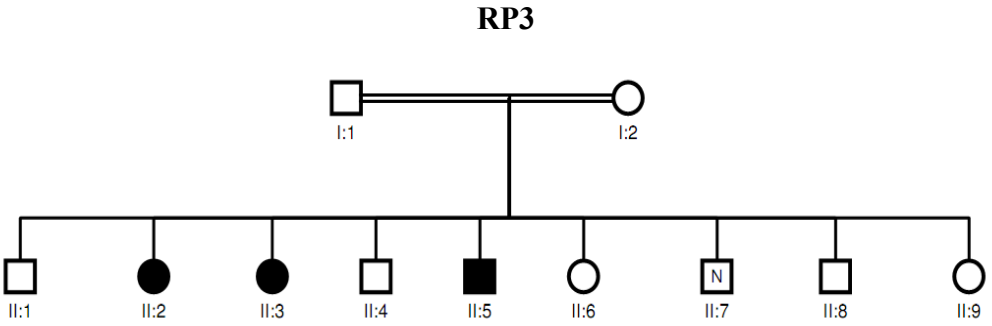

Figure26. Pakistani arRP family (RP3) pedigree.

A consanguineous pedigree with 3 severely affected members. We ruled out known genes.

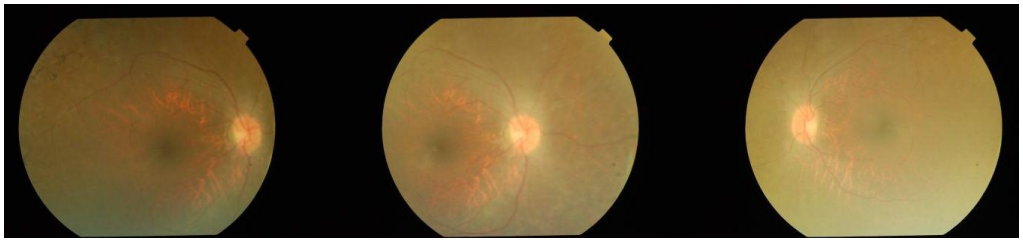

Figure27. Fundus photographs of Pakistani arRP patient (RP3-4).
RP caused by a new RP gene.

We are working on candidate genes for undecided RP patients based on overlap IBD region (Table15) and preliminary RetNet chip and NGS filtration analyses. Summary of preliminary filtration is shown in Table16.

NAME	Chr.	START	END	SIZE (KB)	RANK
RPC6-4	12	83,690,009	90,762,503	7,072.49	9
RPC7-3	12	88,252,256	90,588,877	2,336.62	20
RPC7-4	12	88,252,256	90,588,877	2,336.62	15
RPC13-3	12	88,936,679	90,532,323	1,595.64	28

Table15. Overlap of IBD regions in 3 RP families that might potentially carry the causal gene.

Family	Chr.	Candidate gene	Change
RPC5-4	4	*CC2D2A*	nonsynonymous
RPC7-3	11	*BBS1*	Splicing
	1	*NPHP4*	nonsynonymous
	1	*USH2A*	nonsynonymous
RPC8-6	6	*PRPH2*	nonsynonymous
RPC9-5	2	*LRP1B*	nonsynonymous
	8	*RP1L1*	stopgain
RPC11-3	10	*CDH23*	nonsynonymous
RPC12-2	1	*NPHP4*	nonsynonymous
	6	*PRPH2*	nonsynonymous
RPC13-4	2	*CERKL*	stopgain
RPC 14-5	6	*TULP1*	nonsynonymous
RPC 16-6	10	*CDH23*	nonsynonymous

Table16. Candidate genes for undecided RP patients based on preliminary RetNet chip and NGS filtration.

5. DISCUSSION

In this part of our study, we recruited 40 patients from 14 consanguineous and 2 non consanguineous families diagnosed with RP from Pakistan. Using SNP genotyping by Illumina 10 K chip we discovered, documented and analyzed IBD regions of the patients for known RP genes followed by RetNet chip and NGS. We identified large IBD regions and in these, we detected homozygous reported mutations on *CERKL* and *AIPL1* which have been described before. The mutation causing the phenotype in family RP4 was originally found in Pakistani family (Sohocki *et. al.*, 2000) and was known to cause LCA. We revisited the phenotype and revealed that the phenotype of affected members of RP4 family were comparable to the *AIPL1*-LCA patients previously described (Sohocki *et. al.,* 2000 [77]) and was consists of atrophic macular area, optic nerve pallor and pigmentary changes and was severe than RP1 family with *CERKL* mutation. The studies conducted with Pakistani RP families helped us to confirm one more time how molecular genetic diagnosis is more powerful than clinical diagnosis to determine the right disease diagnosis. The rest of families were checked for all known RP genes located in their IBD regions followed by all the retinal dystrophy genes listed in RetNet website (https://sph.uth.edu/retnet/sum-dis.htm#A-genes) and remained undecided. These families (14) are potential source for new RP gene(s). We have already a very good candidate gene list for some of families created by preliminary NGS filter.

We have also revealed a new region which was overlapped in 3 RP families and might be a potential carrier for new RP gene. Consanguinity will help to narrow

down this region further and NGS candidate gene list will be matched with this overlap region between different families and studied for new RP genes.

CHAPTER 4: CONCLUSIONS

We have recruited, analyzed and evaluated 21 families with childhood blindness from Pakistan with one or several affected members with NCRNA phenotype and 16 families with the RP phenotype. We conducted SNP array following RetNet chip and NGS on the families. According to SNP array and NGS results we successfully genotyped 11 NCRNA families carrying mutation in *LRP5* (4 families), *TSPAN12* (2 families) and *NDP* (2 families) genes and 3 families had mutations in *ATOH7,* two of which (RNC5 and RNC6) have very interesting and reported 6, 523 bp deletion located 20 kb upstream from *ATOH7* gene and is common in North Khorasan, Iran, among descendants of a Kurdish founder population[1] and one mutation which is not reported (c.G125C, p.R42P) (PolyPhen - a tool for prediction of functional effect of human nsSNPs: probably damaging). The rest 10 NCRNA families do not carry any known genes in their IBD regions or all known genes were ruled out indicating that these NCRNA families possibly have new causal gene/s.

We also successfully genotyped 2 RP families with reported mutations in *CERKL* and *AIPL1* genes. The known genes have been ruled out in the rest RP families and NGS results for those families are under investigation. We have identified a very good new candidate gene for RP (segregates within affected family members and parents) which is under study. Summary of the successfully genotyped NCRNA and RP patients are given in Table17.

Family	Chr.	Causal gene	Change	Reported mutations	Co-segregation
RNC2	11	*LRP5*	p. G610R hmz	YES	YES
RNC3	11	*LRP5*	p.D434N hmz	YES	YES
RNC4	10	*ATOH7*	p. R42P hmz	NEW	YES
RNC5	10	*ATOH7*	6, 523 bp deletion hmz	YES	YES
RNC6	10	*ATOH7*	6, 523 bp deletion hmz	YES	YES
RNC7	11	*LRP5*	p.W79R hmz	NEW	YES
RNC9	7	*TSPAN12*	IVS4-2A>G hmz	NEW	YES
RNC11	X	*NDP*	p.C69G hmz	NEW	YES
RNC14	X	*NDP*	c.336_402IVS4del hmz	NEW	YES
RNC17	7	*TSPAN12*	p. P141fsX21 hmz	NEW	YES
RNC19	11	*LRP5*	p. G1401D hmz	YES	YES
RP1	2	*CERKL*	p.R283X hmz	YES	YES
RP4	17	*AIPL1*	p.W278X hmz	YES	YES

Table 17. Successfully genotyped NCRNA and RP families.

CHAPTER 5: REFERENCES

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APPENDIX

Gene	Primer Name	Primer Sequences
SPTBN2	SPTBN2ex14.1fwd	GTAAAACGACGGCCAGTGGGAGGGAAGTCCAAGAG
	SPTBN2ex14.1rev	GGAAACAGCTATGACCATGCAACCAGGCCTCCATGTC
RHOD	RHODex2fwd	GTAAAACGACGGCCAGTCCCCAAGGAGGGAGCAG
	RHODexrev	GGAAACAGCTATGACCATGCCCTGGTTCAGAAGGAAGC
BCAR1	BCAR1ex6.2fwd	GTAAAACGACGGCCAGTACAGTGGTGTGTATGCGGTG
	BCAR1ex6.2rev	GGAAACAGCTATGACCATGGTCCAGGTCCTCAAGGGTG
COL4A2	COL4A2ex23-24fwd	GTAAAACGACGGCCAGTCTGCCAGCTGTGTGAGATG
	COL4A2ex23-24rev	GGAAACAGCTATGACCATGTACTGTTTGCTTCTGGGGTG
SIRPA	SIRPAex3fwd (UCSC)	CATTGATAAACACTTGAGGAAACA
	SIRPAex3rev	GAATGGAGGGTGTTATTATTGAGTT
OVOL1	OVOL1ex2fwd	GGCATCACCTGCAATTACCT
	OVOL1ex2rev	AGCTGGACGCAGGTCTACAT
FAM111B	m13FAM111Bex4.1fwd	GTAAAACGACGGCCAGTCATGCTGACACACCTGTTGA
	m13FAM111Bex4.1rev	GGAAACAGCTATGACCATGACGGCCATCCTTGCATAAG
FAM111B	m13FAM111Bex3fwd	GTAAAACGACGGCCAGTAGAGATGAGGTCCCACACCA
	m13FAM111Bex3rev	GGAAACAGCTATGACCATGATGTGTATGAGCTCCCCAAA
	m13FAM111Bex4.1fwd	GTAAAACGACGGCCAGTAAGACCCTTGTTGAAAATCAAAG
	m13FAM111Bex4.1rev	GGAAACAGCTATGACCATGTCTCACCCTTCAAGGCATAAA
	m13FAM111Bex4.2fwd	GTAAAACGACGGCCAGTTCTCAAGTGCCTGCCTAGTG
	m13FAM111Bex4.2rev	GGAAACAGCTATGACCATGGAGAATTTGCTCTCTGCTGTG
	m13FAM111Bex4.3fwd	GTAAAACGACGGCCAGTTGAAATTAATCACCAGAGTCTGATAC
	m13FAM111Bex4.3rev	GGAAACAGCTATGACCATGATTGTCCCATTGCATGAACC
	m13FAM111Bex4.4fwd	GTAAAACGACGGCCAGTTCTTTCACCAGCTAAGCAATTC
	m13FAM111Bex4.4rev	GGAAACAGCTATGACCATGGAGATCTACCAACCCATCTTGAC
	m13FAM111Bex4.5fwd	GTAAAACGACGGCCAGTCCTGAAGGCCAGATCAAGAA
	m13FAM111Bex4.5rev	GGAAACAGCTATGACCATGGGTCTCATTTGCCAATTTTCAC
ATOH7	ATOH7-20fwd	TCTTACTCATTGCTGTCCCG
5'UTR	ATOH7-20rev	CCAGGTAATCCAGCCAGAGG
	ATOH7-19fwd	GCTTCCATGATGAATGTTTTGGAC
	ATOH7-19rev	AGACCCAGAAATCACCGATG
ATOH7	ATOH7-24fwd	TTCCTCAGAGGCAACCACTC
5'UTR	ATOH7-24rev	GATGCAAAACAGTGTGATTAGTAGG
	ATOH7-21fwd	CTTTTCCACAGTCCTGCATCATTC
	ATOH7-21rev	CCCAGTGTTTCCTAGCTTTGTTAGG
	ATOH7-16fwd	TCTTCAAATGCCAAAGGTCCAC
	ATOH7-16rev	TGGGGGATGATTTCCATGC

Table 18. Primer sequences used for genotyping.

Gene	Primer Name	Primer Sequences
	ATOH7-15fwd	TGGTAGGTAACATCAGGACTGG
	ATOH7-15rev	TTCAGAACAGGAGCAGCGTG
LRP5	seqLRP5ex9fwd	GAGCTGTGTGGCTCACGGCA
LRP5	LRP5 ex6fwd	CCCAGAGGTGTCATGAGGAT
	LRP5 ex6rev	CCAGAATGACAGGTCCAGGT
	LRP5 ex9fwd	TGTACCAGGGGAGAACTTGG
	LRP5 ex9rev	CTTTGAGGCAGGAACAGAGG
BEST1	BEST1EX2 FWD	CAGGGCCTCTGATCCCTAC
	BEST1EX2REV	GTGAACTGGTACACTGGCCC
	BEST1EX3FWD	ATAGCATCGAGGCAGTCCC
	BEST1EX3REV	CAGCTCCTCGTGATCCTCC
	BEST1EX4FWD	AGCAGAAAGCTGGAGGAGC
	BEST1EX4REV	ACCCATCTTCCATTCCTGC
	BEST1EX5FWD	GCAAAGGAGTGCTGAGGTTC
	BEST1EX5REV	CCCAAGGCTTCTCTTTGTAGG
	BEST1EX6FWD	GACCATAGGTACCAGGCCC
	BEST1EX6REV	CTAGGTGAGCCCTTCCCAC
	BEST1EX7 FWD	AAGGCCTTGGTCTCCTGTC
	BEST1EX7REV	TGACACTGCATCCTCGTCTC
	BEST1EX8 FWD	GGGTGTGGAAATAGCAGCAG
	BEST1EX8REV	GCCTCCCTACAGAACAGTGG
	BEST1EX9 FWD	ATTCCTCCAAGTCATCAGGC
	BEST1EX9REV	TGAAGCAACCTGTATGCATTAAC
	BEST1EX10AFWD	CAACTGAGAGAGAGGAGCGG
	BEST1EX10AREV	CTTTGACGGCGCTGATG
	BEST1EX10B FWD	TCAAGTCTGCCCCACTGTATC
	BEST1EX10BREV	ATTGTGGAGGAAGGGTAGGC
	BEST1EX11FWD	CTTTGCCCTCCTACTGCAAC
	BEST1EX11REV	AGCTGTATGGCTGTGACTGG
LCA5	LCA5ex1fwd	CAATGGGAGCTCGGGTAG
	LCA5ex1rev	TCCCTGCTTTAAGAACCACC
	LCA5ex2fwd	TCCTAGGAGTGGTCTCATTTCC
	LCA5ex2rev	TCTGTTCTCGCATTACTGAGG
	LCA5promfwd	TGATCTGACGCTCCTGTGAC
	LCA5promrev	TCACAGCCTGGACAAGTACG
CNGA3	CNGA3ex2fwd	CTGGGTTTGCAGTTACTTTCC
	CNGA3ex2rev	CAACGTGAAGGCATTCTTCC
	CNGA3ex3fwd	CTGACTGTCTCACTCCTGGC

Table18. Primer sequences used for genotyping (cont'd)

Gene	Primer Name	Primer Sequences
	CNGA3ex3rev	CAAATCTTCAACTCCACCCC
CNGA3	CNGA3ex4fwd	AGACAGACAGAGAGGGAGGG
	CNGA3ex4rev	CCAAACAGGATGGAGCAAAG
	CNGA3ex5fwd	AGTGGGATAGGGATTGGGG
	CNGA3ex5rev	TAAGGAGAGAGGCCAAGCTG
	CNGA3ex6fwd	TGAAATTGCCCTAGGCTCTC
	CNGA3ex6rev	CCGAGGGAGAGGTGGAG
	CNGA3ex7fwd	TTACATGATCCAGCGTCTTCC
	CNGA3ex7rev	TAATGTCCCATCCACCATGC
	CNGA3ex8Afwd	GGCCCTCTGTCACTTTTTGA
	CNGA3ex8Arev	GAATCAATCTTGGCCTGGAA
	CNGA3ex8Bfwd	TGGGAACTTGGTCTTGTACATTC
	CNGA3ex8Brev	ATCTGCCGTCCTTTCTCCTC
	CNGA3ex8Cfwd	TTCCAGGCCAAGATTGATTC
	CNGA3ex8Crev	GGCAGTCACACAGCTTTCAA
	CNGA3ex8fwd	TGCATACTGTGTAGCCGTGAG
	CNGA3ex8rev	CCCACATCAGCACCTATCCT
EYS	EYSex11fwd	GGTTTCATCTTAGTAGACAGAGAGGC
	EYSex11rev	CATTGTTACCATGAAACAGTTCG
	EYSex12fwd	TGCACCCCACAACTATCTTC
	EYSex12rev	AATTGCCCAAAGAAGCAATC
	EYSex13fwd	TCATCCTAAGTGGATTAAAGGC
	EYSex13rev	TCTGCCTAAGGAATAATTGGTTG
	EYSex14fwd	GGATATTTTCATTGTTGCTTTGC
	EYSex14rev	TGAATCCAATAAGTGAACAGTTTG
	EYSex15fwd	TGTGGTAAAAGTCATTTGGGG
	EYSex15rev	ATGATTGCGACACCATCTTG
	EYSex16fwd	GCACCACATACTATTAGTTCAAGGG
	EYSex16rev	AGGAGGCCATCATCCCATAG
	EYSex17-18fwd	TTTTAACCTCCATTATGAGTGTATTC
	EYSex17-18rev	ACAAGCACAAATGTATCAGTGG
	EYSex19fwd	CACACATAAAGAACATTTGAGCAG
	EYSex19rev	GGCAGATTATTTCAGGTTTTGG
	EYSex20fwd	GAGAGGTCTTCATTTCTTGGTG
	EYSex20rev	TGTCATCTTAAATGTACTTAGCTCTTG
	EYSex21fwd	TTCACCTGAACTAGGAAAGAAAAG
	EYSex21rev	GACTCTGAAACCTACAGCAAGC

Table18. Primer sequences used for genotyping (cont'd)

Gene	Primer Name	Primer Sequences
EYS	EYSex22fwd	AACAGAGGAAGGAAATGTCAAAC
	EYSex22rev	ACTGGGAAAGCAAAACATGG
	EYSex23fwd	AACTCATTGTCACCCCAAGG
	EYSex23rev	ATGATCATTGCTTAAAACTTGATTAG
	EYSex24fwd	AATGTTAAATGGCACCACGG
	EYSex24rev	AGAGAAGGAGAGATGCGCTG
	EYSex25fwd	GAAATGCAAATGGATCTCAAAAC
	EYSex25rev	CCAATAATGCTTAATTTTACACCCC
	EYSex26Afwd	GCAATTTGCCTGAGATTTATCC
	EYSex26Arev	CAACTGGAGTTGCACTTATGG
	EYSex26Bfwd	TCTGCTACCCCAACGACTTC
	EYSex26Brev	GCTCCCATTAGTGCATACCAG
	EYSex26Cfwd	TTAAGCCAAACATGTGCAAC
	EYSex26Crev	CTGAGCCTGTCAATGGTGG
	EYSex26Dfwd	GGGACCCACTGAGGTACTAAATC
	EYSex26Drev	CGGTGACCATGACAGGC
	EYSex27fwd	CTGGGCTTGAAAGAGGCAG
	EYSex27rev	AAGAGACATCCTGGTGGTGAG
	EYSex28fwd	AAATCTAGGACATATCAGTTGTAAACC
	EYSex28rev	AACCTCAATTTTGAAATGACAAG
	EYSex29fwd	TGCTTCTGGCTTTGTTTTATTG
	EYSex29rev	TTTGAAACCATGCAGATGGC
	EYSex30fwd	TCAAATCAGCCTCCAAATCC
	EYSex30rev	AAACGAAATATAGTGCAGCCTTC
	EYSex31fwd	TTGATCAGTATTGGTTTCATTTTCTC
	EYSex31rev	TGTTTCTTGTTTGTGCTAGTACCC
	EYSex32fwd	TGTACTGGTAGTGAATTATTTGAGGC
	EYSex32rev	ATGCTTCATGCACTGGTCTG
	EYSex33fwd	TCTTTGATGGCAAAGATTGTG
	EYSex33rev	CGCTCCAGACTGGGTGAC
	EYSex34fwd	TGAAAATGTCCACACTTGGC
	EYSex34rev	TCATCCCTAAAGCATGAGTTG
	EYSex35fwd	CATCCAATTACAGAACCAAACATC
	EYSex35rev	TCAGAGGACAATACTGCTGGC
	EYSex36fwd	AAGCTATTAATCTGCTTTCAGTGG
	EYSex36rev	AAGTACCTGCTTGGTGATCAGTC

Table18. Primer sequences used for genotyping (cont'd)

Gene	Primer Name	Primer Sequences
EYS	EYSex37fwd	TGCATCAGCAAAACGTAACC
	EYSex37rev	GCCAATTAGAGTGTCCCTGAG
	EYSex38fwd	GATCTGTAATTCAGAACCAGTCAG
	EYSex38rev	AGGCATCATGGGCTATTCAC
	EYSex39fwd	CAATCCATATAGCTGGTTTGGG
	EYSex39rev	CATGTTATTTCTTCAAAATTCAGGTC
	EYSex40fwd	AAATTTCTCTGCGCATTTCTG
	EYSex40rev	CCTGTCCTCCCATCATGTAAC
	EYSex41fwd	TTTAGGTGGTGGTGTGGGAG
	EYSex41rev	TGAAGTCTTGATCTGGAACTGAG
	EYSex42fwd	GTGTCTGCCACGACAGAAGA
	EYSex42rev	CTGTGATTCCCCTTTGGAGA
	EYSex42fwdshort	CCTACAAGCAACTCTTGGGC
	EYSex42revshort	AGAACATAAAAGCATCAAATGTTTAC
	EYSex43Afwd	TCCAACTTGGCCAGAAACAG
	EYSex43Arev	TGCCATTTACTGTACATTCACCTC
	EYSex43Bfwd	GAATTTGGAGCAAAAGGTGG
	EYSex43Brev	CACAGAGATTCTTTCTCCCAAG
	EYSex43Cfwd	GAAGGTCTAATTGTATGGATGGG
	EYSex43Crev	ACAGTTGATTCCCCGTAAGC
M13fwd		GTAAAACGACGGCCAGT
M13rev		GGAAACAGCTATGACCATG
NRL	NRLex3fwd	GTGAAGAGGGGATGGCAG
	NRLex3rev	CCCCTGTCCCAGTCCATAAC
	NRLex4fwd	GAGCCAGGTAGCGTCGG
	NRLex4rev	TAACGATGCAGAGAACCGTG
EYS	EYSex4.1fwd	TCCTTAACCACAACTTTACTTGG
	EYSex4.1rev	CAACACTCAGCCACTTAGAATTAAC
	EYSex4.2fwd	ATTGGTGGAAGAATGGCATC
	EYSex4.2rev	GAGGATCCCTGATAGGATTTG
	EYSex5fwd	CCTTGGCTAGGGGGTAAGTC
	EYSex5rev	TGGTGAAAAGCATGTGAACTG
	EYSex6fwd	TGATGCTTGAGTAGCTCTATACATTTG
	EYSex6rev	AAAAGGGAAAATACTGATGGAAG
	EYSex7fwd	TGCTGACAGATAATTTCTACTCCAAG
	EYSex7rev	TTAAGTAAAAGTTAGGGTTAAAACCAG

Table18. Primer sequences used for genotyping (cont'd)

Gene	Primer Name	Primer Sequences
EYS	EYSex8fwd	ACAAAGCCTTATTCCTTTTGG
	EYSex8rev	ATATAAGGATATGTTTCTCTGGCTAAG
	EYSex9fwd	GGCTTTTGAACATGGATATGAC
	EYSex9rev	AGATTTCCTAGGATGTAGTTGGTG
	EYSex10fwd	TGTTGGGCTTTGAGGTCTTC
	EYSex10rev	CAAGAAGACTAGGAACAAAATGATTC
KLHL7	KLHLex2fwd	GGGCCTCATTTCCCTTTATG
	KLHLex2rev	TGTGCTGTCATTAGTAAGAGGC
	KLHLex3fwd	GATAAAGCATTTGGCAAGCAC
	KLHLex3rev	AATCTCTTCACTGTTCTGTGGC
	KLHLex4-5fwd	AACATGGCCTGGAATGC
	KLHLex4-5rev	TGTCTTTAAGGTTAAATGCGG
	KLHLex6fwd	CAACTAATTTTCCTGAAGTCATGC
	KLHLex6rev	TCTTTCCCTTGTCATGTGTCC
	KLHLex7fwd	TGAGGTTCTCTCCCTTCAATATG
	KLHLex7rev	CCCTGGCTACTGAGTCTAATCC
	KLHLex8fwd	CCCTCACTTTCCTCAAATGC
	KLHLex8rev	GAAACATTTGGAATCTAAGCCTG
	KLHLex9fwd	GAAACCACACAAGATTAAATTGG
	KLHLex9rev	CAACGACATAAACCTTCAGGG
	KLHLex10fwd	TGACCCTAAATTCTTCCTTCC
	KLHLex10rev	TGGGTTCCTCTGGTCTTTTG
	KLHLex11fwd	TAAAATCCATGTTTTCCCCG
	KLHLex11rev	CAATGACTGGTGACTACTGCG
	KLHLex12fwd	TGGTGGCTATGTGGACATTC
	KLHLex12rev	CCAAACCAAAACTTTGTCACC
CERKL	CERKLex2fwdout	ATTTTTTTTTCAGGTGATTCTAAGTATG
RPE65	RPE65/Ex1/Fwd	GCTCCCAAAGCCATAACTC
	RPE65/Ex1/Rev	AATCAATGCCTTCTCTTCAGG
	RPE65/Ex2/Fwd	GCCCTAGAGTGCCTTCTCTC
	RPE65/Ex2/Rev	GATCCCTCTCCCTGTGACC
	RPE65/Ex3/Fwd	GCCCAATCAGGCTGCTG
	RPE65/Ex3/Rev	AAGTGGGTATATAGGTTGCCTCC
	RPE65/Ex4-5/Fwd	CCCTTTATTCTTCATGTTGTGC
	RPE65/Ex4-5/Rev	ATTTGGAGCTTGGAATGGTC
	RPE65/Ex6/Fwd	TTCAAGGGGTAGTGATGACC
	RPE65/Ex6/Rev	GAGTAATTTAACTATGCACAAAATGC
	RPE65/Ex7-8/Fwd	CAATCAAAATGTGTTTCTTTGCC
	RPE65/Ex7-8/Rev	TCAGCAATATGAAGCCAAACC

Table18. Primer sequences used for genotyping (cont'd)

Gene	Primer Name	Primer Sequences
RPE65	RPE65/Ex9/Fwd	AGTCCAATGAAACCATGGGG
	RPE65/Ex9/Rev	AAACTTCCTACTGCTTTTCCAATTAC
	RPE65/Ex10/Fwd	ATGGCTCTGATACACCTGGC
	RPE65/Ex10/Rev	GCTTTTGCTAAGTCACAGTACTCTTC
	RPE65/Ex11-12/Fwd	TAGCTTCCTGCAGTTCCTCC
	RPE65/Ex11-12/Rev	AGTTTCTTTAGTTTTGACATTCAGC
	RPE65/Ex13/Fwd	TGGCTTGAATCACTTTGTTCC
	RPE65/Ex13/Rev	CAACAAGACCTGATCAAAAGATG
	RPE65/Ex14/Fwd	AAAGAAGTCAGGTCATATGGTTTTC
	RPE65/Ex14/Rev	GCAAAATTGTGCGCATCTG
RPGRIP1	RPGRIP/Ex21/Rev out	CCCTCAGTCTCAATGTTCATC
	RPGRIP/Ex21/Fwd out	CCTATGGTTGATTTCTAGAGAGTAC
	RPGRIP/ex21/Fwd inn nor	CCAGAGGCAGAAGTGATGTC
	RPGRIP/Ex21/Rev inn mut	CCTGTTTTATGTTCTCATCAATC
RDH5	RDH5ex2fwd	GGGAGGGTATTAGGGGAAAG
	RDH5ex2rev	TAGCTTACCACAGCCCTTGC
	RDH5ex3fwd	AGCAGTTTTCAGATGCTCCC
	RDH5ex3rev	TGTGCTGTATCTTAACCACCTCTG
	RDH5ex4fwd	ACTTTCTCAGCTCCCCAACC
RDH5	RDH5ex4rev	CTCCTAGCAGGCTTATGCAG
	RDH5ex5fwd	TGAGGGTGACAAGCCCAG
	RDH5ex5rev	CTTATTTTGTGGCAGGCACC
CNGA3	CNGA3ex2fwd	CTGGGTTTGCAGTTACTTTCC
	CNGA3ex2rev	CAACGTGAAGGCATTCTTCC
	CNGA3ex3fwd	CTGACTGTCTCACTCCTGGC
	CNGA3ex3rev	CAAATCTTCAACTCCACCCC
	CNGA3ex4fwd	AGACAGACAGAGAGGGAGGG
	CNGA3ex4rev	CCAAACAGGATGGAGCAAAG
	CNGA3ex5fwd	AGTGGGATAGGGATTGGGG
	CNGA3ex5rev	TAAGGAGAGAGGCCAAGCTG
	CNGA3ex6fwd	TGAAATTGCCCTAGGCTCTC
	CNGA3ex6rev	CCGAGGGAGAGGTGGAG
	CNGA3ex7fwd	TTACATGATCCAGCGTCTTCC
	CNGA3ex7rev	TAATGTCCCATCCACCATGC
	CNGA3ex8Afwd	GGCCCTCTGTCACTTTTTGA
	CNGA3ex8Arev	GAATCAATCTTGGCCTGGAA
	CNGA3ex8Bfwd	TGGGAACTTGGTCTTGTACATTC

Table18. Primer sequences used for genotyping (cont'd)

Gene	Primer Name	Primer Sequences
	CNGA3ex8Brev	ATCTGCCGTCCTTTCTCCTC
	CNGA3ex8Cfwd	TTCCAGGCCAAGATTGATTC
	CNGA3ex8Crev	GGCAGTCACACAGCTTTCAA
	CNGA3ex8fwd	TGCATACTGTGTAGCCGTGAG
BEST1	BEST1EX2 FWD	CAGGGCCTCTGATCCCTAC
	BEST1EX2REV	GTGAACTGGTACACTGGCCC
	BEST1EX3FWD	ATAGCATCGAGGCAGTCCC
	BEST1EX3REV	CAGCTCCTCGTGATCCTCC
	BEST1EX4FWD	AGCAGAAAGCTGGAGGAGC
	BEST1EX4REV	ACCCATCTTCCATTCCTGC
	BEST1EX5FWD	GCAAAGGAGTGCTGAGGTTC
	BEST1EX5REV	CCCAAGGCTTCTCTTTGTAGG
	BEST1EX6FWD	GACCATAGGTACCAGGCCC
	BEST1EX6REV	CTAGGTGAGCCCTTCCCAC
	BEST1EX7 FWD	AAGGCCTTGGTCTCCTGTC
	BEST1EX7REV	TGACACTGCATCCTCGTCTC
	BEST1EX8 FWD	GGGTGTGGAAATAGCAGCAG
	BEST1EX8REV	GCCTCCCTACAGAACAGTGG
	BEST1EX9 FWD	ATTCCTCCAAGTCATCAGGC
	BEST1EX9REV	TGAAGCAACCTGTATGCATTAAC
BEST1	BEST1EX10AFWD	CAACTGAGAGAGAGGAGCGG
	BEST1EX10AREV	CTTTGACGGCGCTGATG
	BEST1EX10B FWD	TCAAGTCTGCCCCACTGTATC
	BEST1EX10BREV	ATTGTGGAGGAAGGGTAGGC
	BEST1EX11FWD	CTTTGCCCTCCTACTGCAAC
	BEST1EX11REV	AGCTGTATGGCTGTGACTGG
NDP	NDPex3 fwd	GAATTGGCAACGAGTGTGAG
	NDPex3 rev	TTGCCTTAACTCTTTTCTTGCC
	NDP3primerUTRrev	GTGCACCAAACACTGACAGC

Table18. Primer sequences used for genotyping (cont'd)