Study of Clinical Characteristics and Screening of Exon 2 of *PDE6A* gene in Non-Syndromic Familial Retinitis Pigmentosa cases in Pakistan

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Philosophy

In

Molecular Biology

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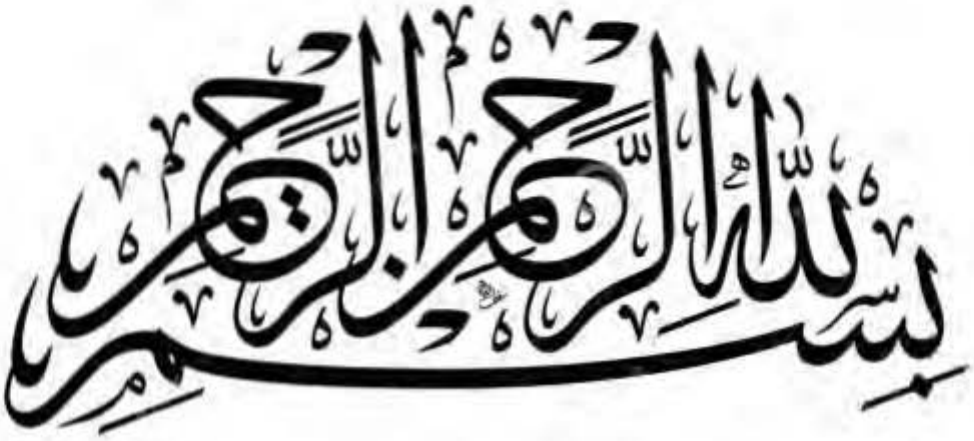

In the Name of Allah, the Entirely Merciful, the Especially Merciful
Al-Fatihah [1: 1], Nobel Quran

CERTIFICATE

This dissertation **"Study of Clinical Characteristics and Screening of Exon 2 of *PDE6A* gene in Non-Syndromic Familial Retinitis Pigmentosa Cases in Pakistan"** submitted by **Nimra Mukhtar**, is accepted in its present form by the Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan as satisfying the thesis requirement for the degree of Master of Philosophy in Parasitology.

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DECLARATION

I hereby declare that the material and information presented in this thesis in my original work. I have not previously presented any part of this work **"Study of Clinical Characteristics and Screening of Exon 2 of *PDE6A* gene in Non-Syndromic Familial Retinitis Pigmentosa Cases in Pakistan."**

Nimra Mukhtar

DEDICATION

With gratitude for the journey, and the companions who walked it with me especially my parents, my supervisor and my friends.

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All praise to the **Almighty Allah (the most merciful and the most benevolent)** and all respect to **His Holy Prophet Hazrat Muhammad (Peace Be Upon Him)** for best owing upon me the courage and energy to accomplish this task.

Firstly, I owe a great deal to my parents for all they have done for me over the years for the completion of this work. My gratitude will not be enough to compensate the continuous support and selfless love of my parents. The credit of all my successes in life goes to unmatchable love and the unremitting prayers of my beloved mother and father.

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LIST OF ABBREVIATIONS

PDE6A	Phosphidiesterase 6 Alpha
RP	Retinitis Pigmentosa
RPE	Retinal Pigment Epithelium layer
ARMD	Age-related macular degeneration
MD	Macular degeneration
RB	Retinoblastoma
DR	Diabetic Retinopathy
RD	Retinal detachment
AIBSES	Acute idiopathic blind-spot enlargement syndrome
VHLD	Von-hippel-lindu disease
TSD	Tuberous sclerosis disorder
VEDF	Vascular endothelial growth factor
DME	Diabetic macular edema
PDR	Proliferative diabetic retinopathy
NPDR	Non-proliferative diabetic retinopathy
BMI	Body mass index
PICCP	Primary inflammatory choriocapillaropathy
ERG	Electroretinogram
TSC	Tuber sclerosis complex
NB	Night blindness
adRP	Autosomal dominant retinitis pigmentosa
arRP	Autosomal recessive retinitis pigmentosa
LCA	Leber congenital amaurosis
DHA	Docosahexaenoic Acid
cGMP	Cyclic guanosine monophosphate
SRP	Syndromic retinitis pigmentosa
NSRP	Non-syndromic retinitis pigmentosa
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction

Abstract

Retinitis Pigmentosa (RP) is a hereditary condition in which the retina of the eye, specifically the photoreceptor layer, degenerates over time. Over 2.5 million people worldwide are thought to be affected with RP. The global prevalence is approximately 1:4000. Eye exam of RP patients indicate weakened retinal vessels, an irregular fundus with an accumulation of bone spicules, a waxy pallor of the optic disc, macular degeneration, and arterial attenuation. The onset age and degree of RP's severity varies, ranging from early childhood to late adulthood and barely perceptible tunnel vision to central vision impairment respectively. RP can be inherited in one of three ways: X-linked RP, autosomal recessive RP, or autosomal dominant RP. Both syndromic and non-syndromic types of RP are possible. According to reports, the most prevalent form of retinal dystrophies in the Pakistani population is autosomal recessive RP. The Al-Shifa Trust Eye Hospital in Rawalpindi, Pakistan, and the Bio-Ethical Review Committee of Quaid-i-Azam University in Islamabad, Pakistan, provided clearance for this study. Sampling was done from August 2022 till February 2023. 20 families with positive family history were collected. An ophthalmologist made the RP diagnosis for all the participating families. Participants were questioned about their family history of illness, a pedigree was drawn, and medical data were gathered. After receiving written authorization, blood samples were taken from both affected and unaffected members. For genetic analysis, genomic DNA was collected. In order to amplify exon 2 of the PDE6A gene for mutational investigation, primers were created. The amplified products were purified and sent for Sanger's sequencing after the amplification of a chosen exon. In this study 20% cases show autosomal dominant pattern of inheritance, 80% cases indicate autosomal recessive mode of inheritance, and no other inheritance pattern was observed. All the RP patients had progressive RP. The ratio of male RP patients was greater than the female RP patients i.e., 57.14% males and 42.86% females. In this study, 20 cases were reported in which 80% (16 families) have non-syndromic RP while 20% cases (4 families) have syndromic RP. The average age of diagnosis of RP patients in this study was 22.64 years and the median was 20 years. 95% families reported in this study has cousin marriages. The findings of this study demonstrated that consanguinity is a factor

causing high incidence of illnesses that are recessively inherited, including RP. Genetic counseling was offered to all participating families as a result.

1. INTRODUCTION

1.1 Structure of Human Eye

The Human Eye is one of the delicate and complex part of the sensory nervous system and its main function is to receive light from the surrounding environment and provide vision (Cioffi. 2020). Eye consists of three main regions known as outer region, middle region, and inner region which collectively allow the eye to perform its function (Willoughby et al., 2010).

The cornea and sclera are found in the outer region. The cornea not only bends and transmits light to the lens and retina, but it also shields the eyes from infection and damage to its deep structures. (Willoughby et al., 2010). The sclera, a layer of protective connective tissue, maintains the form of the eye and shields it from internal and external stimuli. A mucous membrane covers the conjunctiva, which is the portion of the sclera that is visible (Willoughby et al., 2010).

The iris, the ciliary body, and the choroid are all parts of the middle layer. The iris regulates the pupil's size and, consequently, the amount of light that reaches the retina. The ciliary body, which is where aqueous is produced, regulates the lens's size and sharpness as shown in fig. 1.1. The vascular system known as the choroid supplies oxygen and nutrients to the outer layers of the retina. (Willoughby et al., 2010).

Retina and the intricate layer of neurons that capture and process light make up the inner layer. The aqueous, vitreous, and lens were three transparent structures that were enclosed by the ocular layer. (Willoughby et al., 2010).

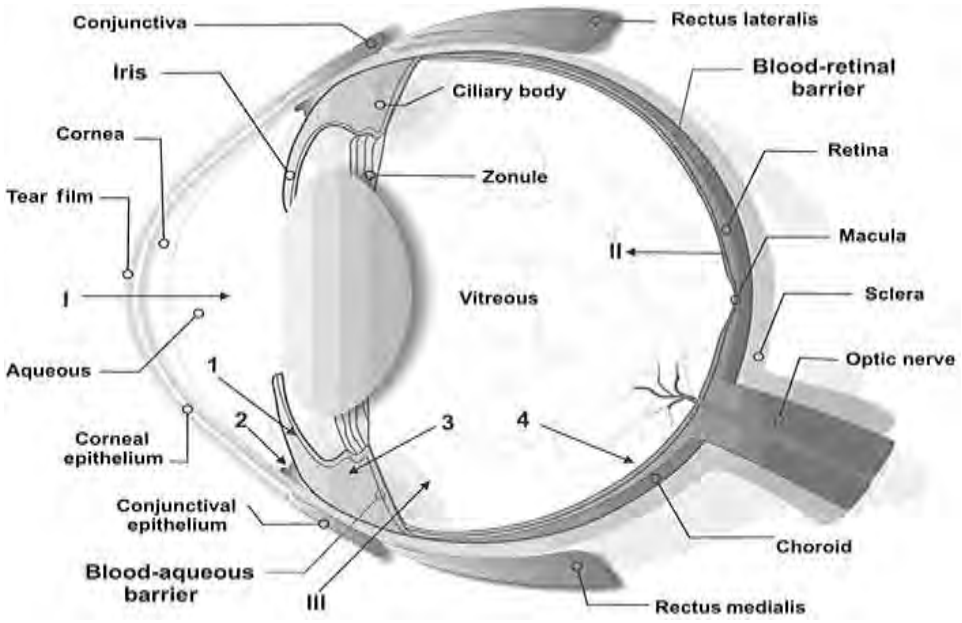

Fig.1.1: Structure of Human Eye

1.2 Anterior Segment of Eye:

The region of the eyeball that is anterior to the lens is called the anterior segment and is made up of the cornea, tear film, trabecular meshwork, conjunctiva, iris, lens, ciliary body, and the anterior section of the sclera. (Williams et al., 2018). The posterior chamber is located between the posterior surface of the cornea and the iris, as shown in fig.1.1; and the anterior chamber is between them. (Cunha-Vaz, J.G. 1997).

Aqueous humor fills the anterior and posterior chambers, which are joined by the pupil's aperture. The aqueous humor's roles include feeding the lens and cornea nutrition and preserving intraocular pressure. It is changed numerous times each day. (Cunha-Vaz, J.G. 1997). The trabecular meshwork controls the outflow of fluid from the eye. A normal tear film and conjunctiva helps in maintaining the homeostasis of ocular surface (Williams et al., 2018).

1.2.1 Posterior Segment of Eye:

The posterior segment of the eye, which makes up the back two-thirds of the eye, contains the vitreous humor, the retina, the choroid, and the optic nerve. (Varela-Fernández et al., 2020). The retina contains photoreceptors that transform light into

electrical signals that are conveyed to the brain via the optic nerve to enable vision as shown in fig.1.1. (Colthurst et al., 2000).

1.2.2 Anatomy of Retina:

The thickness of retina is approximately 0.5mm (Kolb, 1995). The retina is a layer made up of glial cells and photoreceptor cells that is situated in the back of the eye. Incoming light photons are captured by photoreceptor cells, and along their neural routes, glial cells convey electrical and chemical signals to the brain, where these signals are interpreted as a visual image. (Nguyen et al., 2021). It is thought that the 80% of the sensory information is of retinal origin in human (Hildebrand & Fielder, 2011). Neurons present in the retina form 35% of the neurons that enter and exit the brain (Ryan et al., 2013).

Rods and cones are found on photoreceptor cells. Rods are located on the retina's edge and are more sensitive to low light. The retina contains over 100 million rods. Rhodopsin, which is a light-sensitive pigment termed retinal, is the primary component of rod cells. The ability of the retina to absorb photons, which is a function, depends on vitamin A. (Stevens et al., 2015). Cones, which are more sensitive to sunshine, are located at the fovea at the center of the retina. The approximately 6 million cones can also capture wavelengths of colorful light. (Rehman et al.,2022). Three types of cones are present that can detect blue, green, and red wavelengths. The overlap of these wavelengths causes the human to perceive visible light spectrum (Stevens et al., 2015).

The retina is divided into ten layers, with the innermost layers located closest to the pupil and the outermost layers located closest to the eyeball as shown in fig.1.2:

Inner Limiting Membrane: It serves as a supple barrier to the vitreous fluid and primarily preserves the homeostasis of the retina. (Heavner & Pevny, 2012).

Retinal Nerve Fiber Layer: Astrocytes and retinal ganglion cells are combined together to form this layer. The basal lamina for the retinal nerve fiber layer is the inner limiting membrane**.** (Jonas & Dichtl, 1996).

Ganglion Cell Layer: The cell bodies of these layer's ganglion cells' axons combine to form the optic nerve**.** (Nguyen et al., 2021).

Inner Plexiform Layer: This layer is where bipolar cell axons synapse onto ganglion cells, and its primary purpose is to modulate electrical transmission between bipolar cells and ganglion cells. (Hartveit & Veruki, 2012;Tanaka & Tachibana, 2013).

Inner Nuclear Layer: Amacrine cells, bipolar cell bodies, and horizontal cells make up this layer. Bipolar cells transfer different synaptic signals from photoreceptor cells onto ganglion cells. (Euler et al., 2014). Feedback modulation onto rod and cone cells is provided by horizontal cells (Masland, 2012).

Outer Plexiform Layer: The dendrites of the cells that make up the inner nuclear layer form synapses here with the projections from the photoreceptor cells. (Nguyen et al., 2021).

Outer Nuclear Layer: This layer contains the rod and cone cell bodies. (Nguyen et al., 2021).

External Limiting Membrane: Cone and rod cell bodies are divided from their inner and outer segments by a membrane. There are gap connections between Muller cells and photoreceptor cells. (Nguyen et al., 2021).

Photoreceptor Layer: The layer is made up of the inner and outer cone and rod segments. The inner layer has a pool of mitochondria necessary to fulfill the high metabolic demands of photoreceptor cells, and the outer segment contains rhodopsin necessary for phototransduction.(Narayan et al., 2017).

Retinal Pigment Epithelium: The retinal pigment epithelium, or RPE, is the outermost retinal layer and is situated next to the choroid layer between the neural retina and the Bruch membrane. The blood retinal barrier is aided by RPE along the endothelium of the retinal arteries. RPE also has the capacity to transport water and ions, as well as to secrete cytokines and growth factors (Spencer et al., 2017). In RPE, all-trans-retinal is recycled into 11-cis-retinal and delivered back to the rods and cones where it is employed repeatedly in the phototransduction process. (Strauss, 1995).

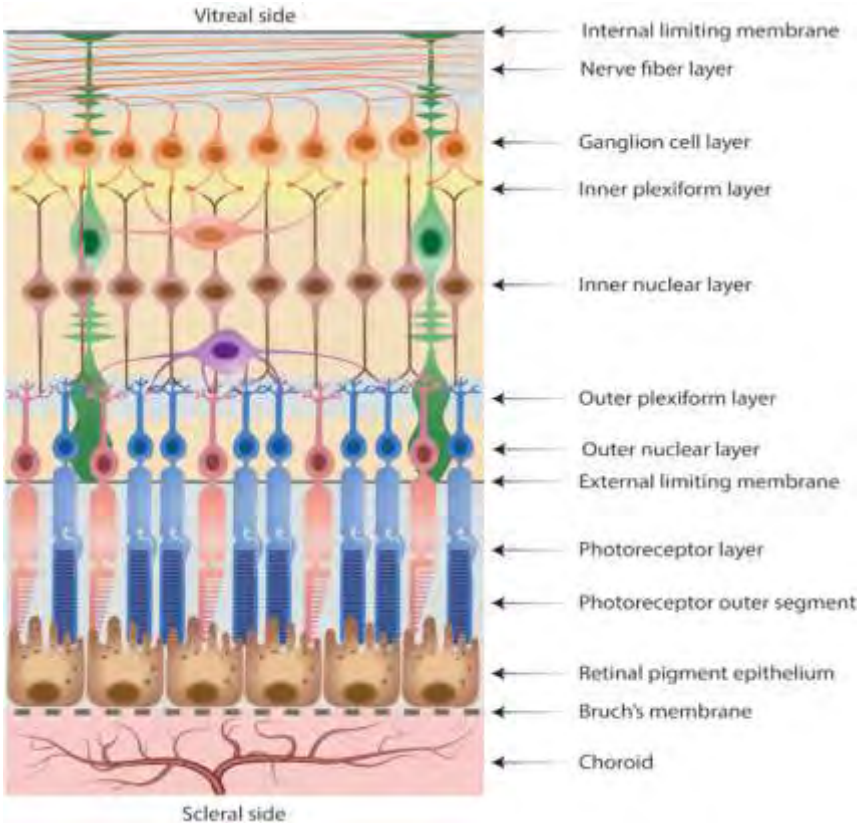

Figure 1.2: Layers of Retina in Human Eye

The most sensitive region of retina is macula which provide the highest visual acuity. It contains the pigments called lutein and zeaxanthin. Th ese pigments has blue-light filtering properties and anti-inflammatory characteristics (Jia et al., 2017). Research indicates that the dietary supplementation of these pigments reduces the risk of retinopathy in infants born pre-term and diabetic retinopathy in adults. The central region of macula is known as fovea. Fovea consists of high concentration of cones (Gong & Rubin, 2015).

The principal pathway via which photoreceptor cells of the retina gather visual information and transmit it to the brain is the optic nerve. The blind spot, an area lacking rods and cones, is where the ganglion cells that make up the optic nerve's axons converge as shown in fig.1.3 (Smith & Czyz, 2023).

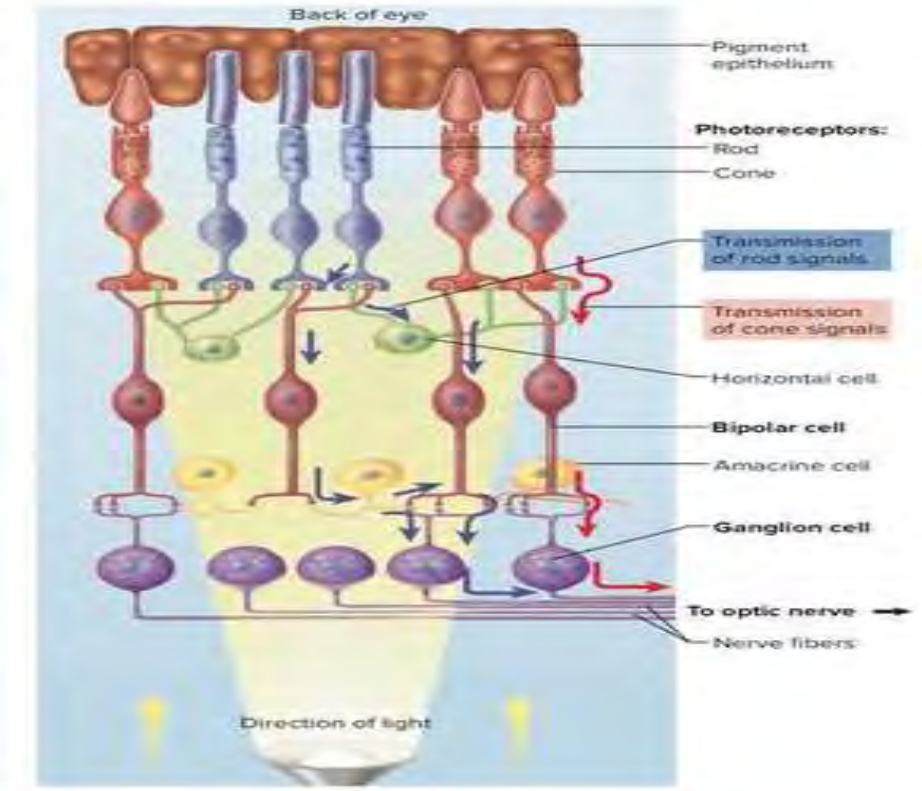

Figure 1.3: Structure and Distribution of Retinal Cells

1.3 Retinal Disorders:

Patients having retinal disorders have major symptom of visual loss. This vision loss occurs due to any defect in the visual pathway e.g., cortical disorders, optical aberrations, or functional visual loss (Landau & Kurz-levin, 2011).Some of the most important retinal disorders are:

i. Diabetic Retinopathy
ii. Retinoblastoma (RB)
iii. Age-related Macular Degeneration (AMD)
iv. Acute Idiopathic Blind-Spot Enlargement Syndrome (AIBSES)
v. Retinal Detachment (RD)
vi. Tuberous Sclerosis (TS)
vii. Von Hippel-Lindau Disease (VHLD)
viii. Cone Dystrophy
ix. Retinitis Pigmentosa (RP)

1.3.1 Diabetic Retinopathy:

It results from the elevated blood sugar level and is present in both types of diabetes. In developing countries, the age group of adults under 75 years of age having diabetes blindness is the most common complication (Williams et al., 2004). Development and progression of retinopathy is strongly predicted by the duration of diabetes (Fong et al.,2003). Based on proliferation, diabetic retinopathy can be classified in to two stages. In non-proliferative diabetic retinopathy, the degeneration of retinal microvasculature occurs which leads to the leakage of plasma through the compromised blood retinal barrier and microaneurysms. Macular edema is caused due to fluid accumulation that results in the loss of visual acuity (Cogan et al., 1961). Proliferative diabetic retinopathy is characterized by neovascularization due to ischemia and artery occlusion. Blood is leaked in the vitreous because the vessels are fragile. This leaked blood block the light from hitting the fovea and scarring leading to tractional retinal detachment (Williams et al., 2004).

In the first 20 years of the disease, retinopathy affects almost all patients with type 1 diabetes and 60% of individuals with type 2 diabetes. According to the Wisconsin Epidemiologic Study of Diabetic Retinopathy, diabetic retinopathy is to blame for 86% of blindness in cases with younger onset and for one-third of cases with older onset. (Fong et al.,2003).

1.3.2 Retinoblastoma (RB):

Cancerous growth grows in a young child's neural retina in retinoblastoma. It happens as a result of a loss-of-function mutation in the tumor suppressor gene Rb1. (Mendoza & Grossniklaus, 2015). It accounts an estimated 3% of all pediatric cancers (Rao & Honavar, 2017). One new instance of retinoblastoma was reported every 15,000–20,000 live births, or 9000 new cases each year. In underdeveloped nations, retinoblastoma accounts for 70% of deaths. (Kivelä, 2009).

Leukocoria is the most typical early indication of retinoblastoma and is visible while the tumor is still inside the eye as shown in fig.1.4. Inflammation, glaucoma, strabismus, and poor visual tracking are some additional symptoms of retinoblastoma (Abramson et al., 1998). Retinoblastoma is brought on by two Rb1 gene mutational events. In the hereditary form of retinoblastoma, one mutation occurs in the somatic cells and the other is inherited in the germline cells. When retinoblastoma is present in

a nonhereditary type, both mutations take place in somatic cells. Every unilateral bilateral and multifocal form is inherited.(Knudson, 1971).

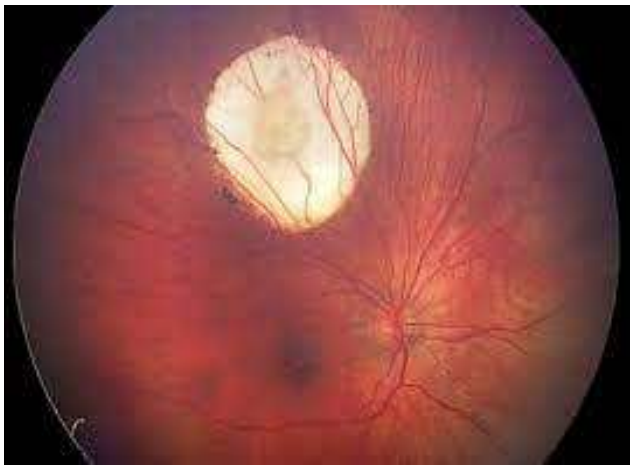

Figure 1.4. Fundus Photograph of retinoblastoma patient

1.3.3 Age related Macular Degeneration (AMD):

Age-related macular degeneration (AMD) is the most common cause of irreversible severe vision loss in adults over 50 in the US (Bressler et al., 1988). More than 9 million Americans currently have AMD, and by 2050, that figure is projected to increase to 17.8 million. (Rein et al., 2009). It is a neurodegenerative condition that affects the interface between the neuronal retinal layers and the retinal pigment epithelium (RPE). There are two categories of AMD: dry and moist. RPE lesions that generate amorphous deposits known as drusen between the RPE and the Bruch membrane are a hallmark of dry AMD. Atypical neovascularization that extends from the choroid and penetrates the Bruch membrane characterizing moist AMD. (Zając-Pytrus et al., 2015).

The prominent risk factor for AMD is increased age (Chakravarthy et al., 2010). Other risk factors are broadly classified into environmental, sociodemographic, ocular, and systemic factors (Gheorghe et al., 2015). Daily activities such as driving, reading, and recognizing faces in both types of AMD. An estimate show that the one-third of patients with advanced AMD suffer from depression (Casten et al., 2004).

1.3.4 Acute Idiopathic Blind-Spot Enlargement Syndrome (AIBSES):

Acute Idiopathic Blind-Spot Enlargement Syndrome (AIBSES) is a rare outer retinopathy(Liu et al., 2014). AIBSES clinical features include visual field defects, photopsia, abnormal results of focal electroretinography, and abnormal findings from fundoscopic and fluorescein angiography. The peripapillary retina is affected by AIBSES and causes an afferent pupillary defect. AIBSE is distinguished from numerous evanescent white dot syndrome by its conspicuous preference for the peripapillary retina. Patients with AIBSE did not experience visual field recovery (Volpe et al., 2001). Optical Coherence Tomography (OCT) and Visual field examination are the major means of detecting ABISES (Liu et al., 2014).

1.3.5 Retinal Detachment (RD):

The term "retinal detachment" (RD) refers to the separation of the retinal layers between the retina's inner neuronal layers and its pigment epithelium, which results in ischemia and ensuing photoreceptor degradation. RD is typically an emergency of the eye, and treatment and early discovery can prevent permanent vision loss. Age, trauma, myopia, diabetic retinopathy, cataract surgery, and these conditions are some of the main risk factors for retinal detachment. (Pokhrel & Loftus, 2007).

There are three different kinds of retinal detachments: exudative, tractional, and rhegmatogenous. Rhegmatogenous is the most frequent of the three, distinguished by a retinal tear through which liquified vitreous seeps beneath the retinal layers. (Feltgen & Walter, 2014). 1 in 10,000 people a year has been diagnosed with rhegmatogenous retinal detachment (Steel, 2014). Less frequently occurring tractional retinal detachment is caused by retinal scarring, which is typically seen in diabetic retinopathy. Physical disruption of the subretinal space due to fluid accumulation and blood-retinal barrier collapse without retinal tears is known as exudative retinal detachment. It may also be brought on by conditions like intraocular tumors and age-related macular degeneration. (Amer et al., 2017).

1.3.6 Tuberous Sclerosis (TS):

Tuberous sclerosis (TS) is a multisystem, autosomal dominant illness that can affect both children and adults. TSC1 or TSC2 gene mutations are responsible for its occurrence. (Crino et al., 2006). It was first described by Bourneville in 1880. 2

million people worldwide are estimated to be affected by this disease (Henske et al., 2016) Retinal phakomata, which can be white and calcified, granular, or flat transparent and noncalcified, are one of the main ocular manifestations. Polio, depigmented retina and iris, angiofibromas, and abnormal colobomata are further findings. With time, further ocular and systemic symptoms of TD could appear. (Williams & Taylor, 1985). In 50-70% of patients diagnosed with TD flat hamartomas occurs(Rowley et al., 2001). Other organs effected in this disease include eye, kidney, heart, brain, bones and lungs (REED et al., 1963).

1.3.7 Von Hippel-Lindau Disease (VHLD):

An inherited disorder, von Hipple-Lindau disease (VHLD). The prevalence of VHLD is 1 in 36000 live births. Patients experience both benign and malignant tumor development, including hemangioblastomas of the retina and central nervous system, pheochromocytomas, pancreatic neuroendocrine tumors, clear cell renal cell carcinomas (RCC), and endolymphatic sac tumors. It is brought on by the VHL gene's germline loss of function, which affects one allele at chromosome 3 arm p. When second hit occurs tumor forms due to the loss of the other allele (Chittiboina & Lonser, 2015). For the differential diagnosis of a patient with cerebellar hemangioblastoma VHLD must be always. Timely diagnosis of the syndrome is necessary for the manifestations such as retinal hemangioblastoma or renal carcinoma (Violaris et al., 2007).

1.3.8 Cone Dystrophy:

The cone dysfunction syndromes are a variety of inherited, stable retinal conditions with diminished central vision, variable degrees of color vision problems, nystagmus, and photophobia. (Aboshiha et al., 2016). It usually starts in the mid-teenage years or later in life. The estimated prevalence of this group of disease is 1 in 30,000-40,000 individuals. Visual acuity deteriorates gradually to 20/200 or even counting fingers (Tsang & Sharma, 2018). It has varying mode of inheritance and have been classified in to stationary and progressive cone dystrophies (Michaelides et al., 2004).

1.3.9 Retinitis Pigmentosa:

A disease condition was first identified and named by Dr. Donders in 1857 (Mandal et al., 2023), although four years ago his colleague A.C. Van Trigt provided the first description of retinitis pigmentosa using ophthalmoscope (Van Trigt., 1852). Retinitis pigmentosa (RP) is a term used to describe a group of retinal degenerative illnesses marked by the gradual loss of photoreceptors and the retinal pigmented epithelium (RPE). Hereditary retinal dystrophies are another name for Rp.(O'Neal & Luther, 2023). On a global scale, ~1 in every 4,000 persons is impacted by RP and around 1.5 million individuals were affected by RP globally (Pagon, 1988). Depending on the geographic location reports vary from 1:9000 (Na et al., 2017) to as high as 1:750 (Jonas et al., 2009). It is one of the most common retinal degenerations causing visual impairment in all age groups. Till now 69 genes have been identified and mapped to cause RP (Cater et al., 2006).

1.3.9.1 Pathophysiology of Retinitis Pigmentosa:

Photoreceptors of both rods and cones are affected in RP but the type of genetic defect determines which cells are more affected. Mostly rod photoreceptors were malfunctioned in patients diagnosed with RP (Mandal et al., 2023). The first symptom to occur is the rod photoreceptor dysfunction followed by the cone photoreceptor dysfunction (Yang et al., 2021). The typical symptoms of RP include night blindness, progressive loss of peripheral vision, tunnel vision, and even blindness is observed in advanced stages of RP (Q. Gao et al., 2019). One of the macular conditions related to RP is cystoid macular edema (CME) causing the loss of central vision (Fahim et al., 1993). It has been reported that 50% of patients having RP also has CME Other macular complications such as macular hole and epiretinal membrane are also observed in RP patients (Strong et al., 2017).

1.3.9.2 Types of RP:

Based on mode of inheritance pattern RP is categorized as sporadic, autosomal dominant (adRP), autosomal recessive (arRP), X- linked, mitochondrial and digenic (Kajiwara et al., 1994). If the disease is affecting only eyes, then it is known as "non-syndromic RP". When RP occurs in conjunction with other systemic diseases then it is referred as "syndromic RP" (Wolfrum & Nagel-Wolfrum, 2018). Milder symptoms

appear in the RP patients whose family history indicate autosomal dominant mode of inheritance as compared to X-link inheritance whose patients show severe symptoms with loss of central vision (Mandal et al., 2023).

A. Syndromic RP:

When RP occurs in conjunction with other systemic diseases then it is referred to as "syndromic RP" (Wolfrum & Nagel-Wolfrum, 2018). The three common forms of syndromic RP are Usher syndrome, Bardet- Biedl Syndrome (BBS), and Cohen Syndrome (Makiyama et al., 2014).

The most common form of Syndromic RP is Usher syndrome, which takes its name from ophthalmologist Charles Usher who first describes 69 patients having this syndrome (Castiglione & Möller, 2022). It is characterized by retinopathy (RP), hearing loss, and vestibular areflexia with different onset and entities(Koenekoop et al., 1993; Reiners et al., 2006). The global estimation of this syndrome with low average is 1/30,000 people because several other studies reported the prevalence that varies from 1 to 17 per 10,000 people (Espinós et al., 1999). Bardet-Biedl Syndrome (BBS) prevalence varies markedly between populations from 1/160,000 to 1/13,500 and 1/17,5000 (Farag & Teebi, 1989; Green et al., 1989). Patients with BBS in addition to retinopathy (RP), they have symptoms of postaxial polydactyly, obesity, renal dysfunction, hypogonadism, and/or cognitive impairment (Mockel et al., 2011).

B Non-Syndromic RP:

There are no associated symptoms in Non-Syndromic RP. Without any complications, the disease is diagnosed by the common signs and symptoms (Nwosu et al., 2020). There are about 65-75% of reported cases of non-syndromic RP. In last 3 years about 6500 non-syndromic RP cases had been reported in US. Among all reported cases 20% are of arRP, 30% of adRP, 15% are of X-linked RP, 55% are of recessive LCA and the remaining are of isolated RP (O'Neal & Luther, 2022). It is represented in Table 1.1. Autosomal recessive mutations were mostly observed in isolated cases but de novo mutations such as dominant cases are also reported in patients.

1.3.9.3 Prevalence:

Around the globe, 285 million people are suffering from visual impairment. Amon them 2-3% are affected with Retinitis Pigmentosa (Colombo et al., 2021). This

disease has a worldwide prevalence. More than 2.5 million people are affected from RP worldwide. Depending upon the demography of area, it's prevalence ranges from 1:4000 to 1:9000 (Toms et al., 2020;F.-J. Gao et al., 2019).

Table 1.1: Estimated percentages of RP Types (Ferrari et al., 2011)

Category	**Type**	**Percentage (%)**
Non- syndromic RP	Autosomal Dominant RP	20-25
	Autosomal Recessive RP	15-20
	X-Linked RP	10-15
	Leber congenital amaurosis	4
	Digenic RP	Very rare
Syndromic RP	Usher Syndrome	10
	Bardet-Biedl Syndrome	5

In past decades, a rapid increase in cases of RP has been reported. In 2010, the number of RP cases reported in South Asia are, 58,528 cases in Pakistan, 58,963 cases in Bangladesh 391,570 cases in India, 15,482 cases in Afghanistan, 9318 cases in Sri Lanka, 1603 cases in Bhutan (Bouzidi et al., 2021;Sun et al., 2021;Thapa et al., 2020). In 2020, an increase in these numbers have been reported such that, 92,762 cases in Pakistan, 83,293 cases in Bangladesh, 588,320 cases in India, 21,821 cases in Afghanistan, 13,931 cases in Sri Lanka, 3887 cases in Bhutan (Karali et al., 2019;Van Cauwenbergh et al., 2017). (Cauwenbergh et al., 2017; Bouzidi et al., 2021)

Autosomal recessive RP is the most common form of retinal dystrophies in Pakistani population (Moore et al., 2020). According to studies on the population of Pakistan, autosomal recessive conditions such as Leber Congenital Amerosis, 10% autosomal recessive cone-rod dystrophy, 8% autosomal recessive congenital stationary night blindness, and 64% autosomal recessive retinitis pigmentosa account for these conditions (Maria et al., 2015;Shahzad et al., 2013). In total, more than 60 genes and 150 mutations are reported to be associated with RP worldwide. As represented in fig.1.5; there are many genes that overlap between RP and other retinal dystrophies. Most frequently reported genes associated with RP in Pakistani population are *SEMA4A, LCA5, RPGRIP1, RP1, TULIP1, AIPL1, PDE6B, PDE6A,* and *CRB1* (Kannabiran et al., 2022;Zafar et al., 2017). Different mutations found in Pakistani population were represented in table 1.2.

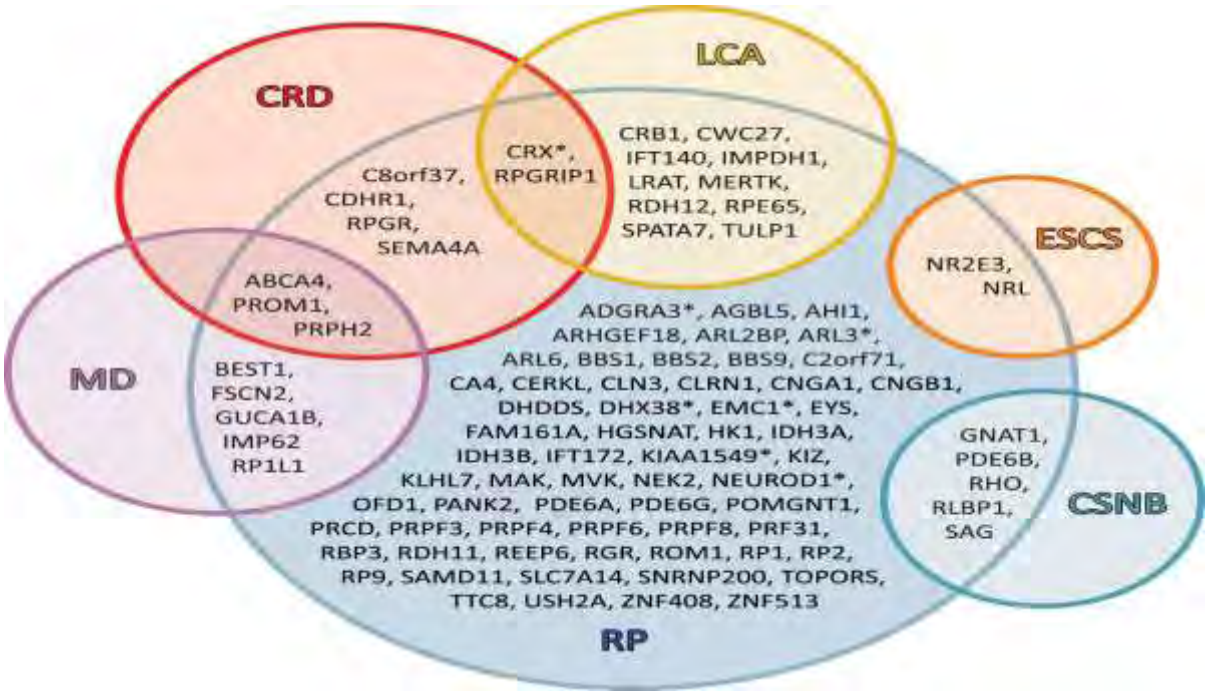

Fig. 1.5. Venn diagram showing genetic overlap between RP and other inherited retinal dystrophies (Verbakel et al., 2018)**.**

1.3.9.4 Clinical Findings, Signs and Symptoms:

The loss of cone photoreceptors is followed by the primary degeneration of the rod photoreceptors, which defines RP. (Verbakel et al., 2018). The gradual deterioration of visual field is the defining hallmark of RP. The bilateral symmetry of this visual field reduction is very strong. (Massof et al., 1979). Nyctalopia, or night blindness that

makes it difficult to adapt to the dark, is one of the typical symptoms of RP. This condition is followed by a decrease in visual fields, which causes tunnel vision and finally results in legal blindness or, in most cases, complete blindness (Hartong et al., 2006).

Nyctalopia, or night blindness that makes it difficult to adapt to the dark, is one of the typical symptoms of RP. This condition is followed by a decrease in visual fields, which causes tunnel vision and finally results in legal blindness or, in most cases, complete blindness (Verbakel et al., 2018). Most patients of RP retain their ability to perceive light due to residual macular function or/and the presence of a preserved peripheral temporal retinal island (Hamel, 2006). Photopsia is an often neglected and common symptom in RP patients that can be highly disturbing to patients (Heckenlively et al., 1988). Photopsia is usually noticeable in people with more severe stages of RP, while it can occur in early phases of the disease as well (Bittner et al., 2009;Bittner et al., 2011). The visual hallucinations in RP can take animate forms, which corresponds with the diagnosis of Charles Bonnet syndrome (O'Hare et al., 2015). RP patients can also experience dyschromatopsia and photophobia (Pinckers et al., 1993).

Additionally, frequent in patients with RP nystagmus that manifests early is refractive error linked to the illness. Cystoid macular edema (CME), epiretinal membrane development, and macular holes are a few examples of macular problems. 50% of RP patients have been reported to have CME. (Strong et al., 2017).

1.3.9.5 Age of Onset and progression:

The age of onset refers to the age at which the patient has reported the visual symptoms. The age of onset of RP, varies and depends upon its type (Verbakel et al., 2018;Verdina et al., 2021). Although RP patients do not exhibit any obvious symptoms when they are young, the progressive degeneration of the photoreceptor cells causes them to gradually lose their vision as they age. (Hartong et al., 2006). Patients with RP were diagnosed at an average age of 35.1 years, while the median age was 36.5 years. One year old was the youngest person, and the oldest person was 89. (Tsujikawa et al., 2008). If the symptoms appear earlier, it indicates the rapid progression of RP. Inheritance pattern also paly important role in the severity of the disease (Kuehlewein et al., 2020). The median age of patients with RP was 36.5 years,

while the average age of diagnosis was 35.1 years. The youngest person was one year old, while the oldest was 89 (Bunker et al., 1984) with respect to retaining central vision have the best long term prognosis (Grover et al., 1996;Hamel, 2006).

1.3.9.6 Diagnosis

RP can be diagnosed based on retinal findings, clinical symptoms, vision assessment, fundus examination and ERG (Electroretinogram) (Cundy et al., 2021). The retina also shows some degree of constructed retinal vessels, optic nerve pallor and bony spicule pigmentation in peripheral region of retina (Khan et al., 2021). At initial stages, the defect in the visual field starts from the peripheral region and in final stages of RP it progresses towards the central region (Chatterjee et al., 2021). The most efficient tool used for the diagnosis of RP is ERG. It tells us about the extent and number of affected and normal retinal cells (de Bruijn et al., 2020). Depending on the intensity of the light stimulus the response of the photoreceptor cells is measured in the form of electrical signal. In bright light, mainly cones are contributing to the electrical signals and in dim light, rods are contributing to electrical signals as indicated in fig.1.6. This variation provides the basis for ERG diagnosis(Arsiwalla et al., 2020)

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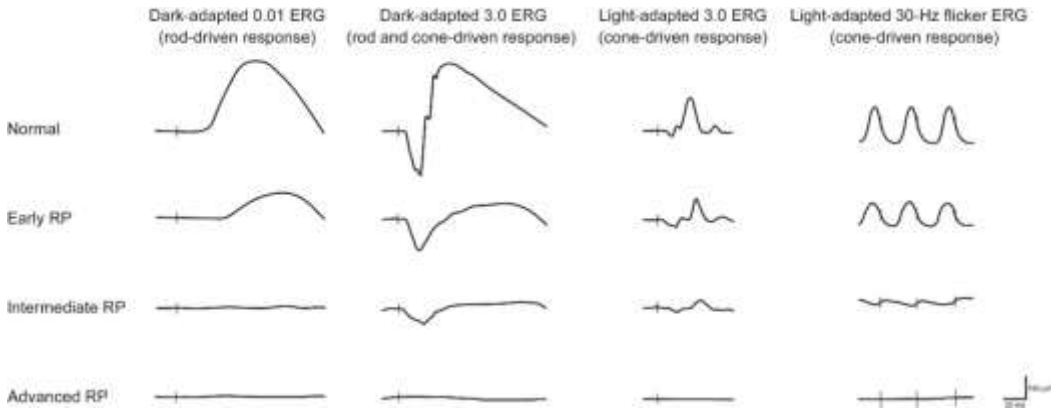


Fig 1.6: ERG representation at different stages of Retinitis pigmentosa (Verbakel et al., 2018)

1.3.9.7 Treatment:

No standard treatments for patients with Retinitis pigmentosa are available. For many years the most widely recommended treatment is supplementation of Vitamin A,

which slows down the progression of the disease (Shintani et al., 2009). Numerous therapeutic options, such as antiapoptotic drugs, gene therapy, neurotrophic factors, and nutritional supplements, are being investigated based on our understanding of the pathophysiology of degeneration of the retina as acquired from studies on animal models. For patients with end-stage RP, retinal implant technologies continue to provide the possibility of eyesight. (Musarella & MacDonald, 2011).

i. Pharmacological Medication:

Several Pharmacological medications of RP which slow down the disease progression are stated below.

- Acetazolamide
- Oral Nilvadipine
- Brimonidine 0.2% Eye Drops
- Oral Valproic Acid
- Beta-Carotene Acid Supplementation
- Lutein Supplementation
- Docosahexaenoic Acid (DHA) Supplementation
- Vitamin A and D Supplements

ii. Retinal Implant:

An implant known as "Argus II" is available in USA for the treatment of RP. It is implanted into one eye and is paired with the glasses having camera (Verbakel et al., 2018). In the form of electrical signals, the received images are then transferred to the retina. It helps the patients with end stage RP. The ability to identify people, locate light, and to read some letters improve at 9 inches distance (Cornford et al., 2017).

iii. Glasses:

To cope with the vision impairment in RP special glasses have been designed (Dias et al., 2018). These glasses help to improve vision and help to minimize the light intensity (Jin et al., 2019). To correct the vision impairment glasses are prescribed to all the RP patients (MacPherson et al., 2017).

iv. Lifestyle and Dietary Modifications:

Lifestyle modifications also impart a significant role in the treatment of retinitis pigmentosa. It includes following precaution measures:

- Walk in green lawn with bare feet for 30-60 minutes.
- Good lighting conditions in all working areas especially in reading area.
- More use of green vegetables and fruits as they are the rich source of Vitamin A and D.

v. Genetic Counseling:

Retinitis Pigmentosa is a genetic disorder inherited from their parents. The most common form is the autosomal recessive RP (Valle et al., 2021). If both parents are carrier or affected, then the offspring will be affected. If one parent is affected and other is normal, then the offspring is carrier and passed the disease to the next generation if married to a carrier or affected one (Wheway et al., 2020). RP occurs mostly in families having cousin marriages and passed within generations. If a couple has both parents affected, then they must be advised to go for family planning or no more kids (Xu & Wang, 2017).

1.4 PDE6A gene and its association with RP:

Phosphodiesterase 6 Alpha (PGE6A) gene is located on the long arm or q: arm of chromosome no 5 at position 32 i.e., Chr.5q32 (Bujakowska & Comander, 2020). It has 21 introns and 22 exons represented in fig.1.7. It is approximately 45-50 kb in size (Dawood et al., 2021). These exons code for the long protein of 860 amino acids (Li et al., 2022). 40 pathogenic mutations have been reported in PDE6A gene worldwide. Among them, 26 mutations are single base substitution which constitute about 65% of all pathogenic mutation of PDE6A(Occelli et al., 2017; Nair et al., 2017). The remaining 14 variants are missense and non-sense variants. Mutations in PDE6A gene causes malfunctioning of cGMP (Cyclic Guanosine Mono phosphate) and leads to retinal degeneration ultimately associated with autosomal retinitis pigmentosa in humans(Khan et al., 2021).

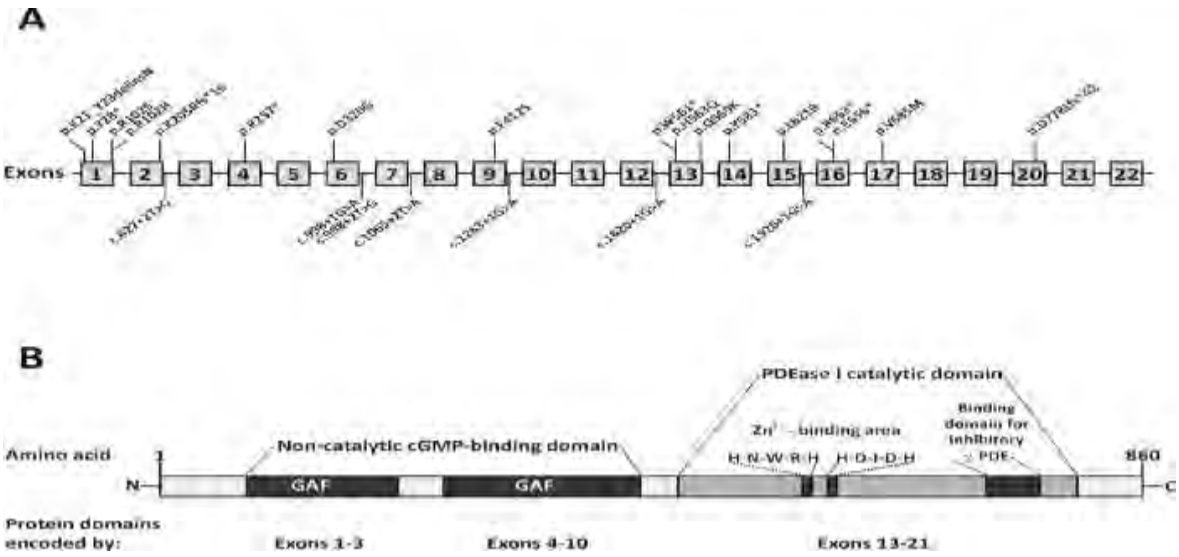


Fig 1.7. Genomic and protein Structure of PDE6A and Location of Variants (Kuehlewein et al., 2022**).**

The phosphodiesterase 6 (PDE6A) enzyme is hetero tetrameric protein which consists of alpha, beta, and two gamma subunits (Takahashi et al., 2018). The alpha subunit is coded by PDE6A gene, beta subunit is coded by PDE6B gene, and gamma subunit is coded by PDE6G gene (Schön et al., 2017). The alpha subunit has mass of 88kDa, beta subunit has mass of 84kDa, and gamma subunit has mass of 11kDa (Crouzier et al., 2021). These three subunits are the essential part of cyclic guanosine monophosphate (cGMP).

Usually, the genetic mutations initiating RP leads to the elevation of cyclic guanosine monophosphate (cGMP) due to the disturbance in the phototransduction cascade (Arango-Gonzalez et al., 2014). cGMP levels increase in the dark due to high guanylate cyclase activity (Olshevskaya et al., 2002). Light induces conformational changes in opsin molecules, leading to the sequential activation of transducin and PDE6, the process known as phototransduction. High cGMP levels cause the cyclic-nucleotide-gated (CNG) cation channels to retain their open state, allowing Ca2+ influx (Michalakis et al., 2005). cGMP levels reduces when PDE6 is activated, CNG channels become closed causing hyperpolarization and signal are transmitted to second order neurons as shown in fig.1.8. cGMP became excessive when genetic mutations affect the PDE6 gene function and subsequently leading to the rod photoreceptors death (Farber & Lolley, 1974; Sahaboglu et al., 2013), followed by a secondary death of cone photoreceptors which is mutation independent (Hamel,

2006). PDE6 has two catalytic subunits, alpha and beta, and two inhibitory gamma subunits when it is inactive in rod photoreceptors. When all three PDE6 subunit mutations are considered collectively, they account for up to 4-8% of human RP patients. (Bayés et al., 1995; Dryja et al., 1999; Dvir et al., 2010). Many PDE6A mutations are known to cause RP but, so far, they have been relatively little studied (Sakamoto et al., 2009).

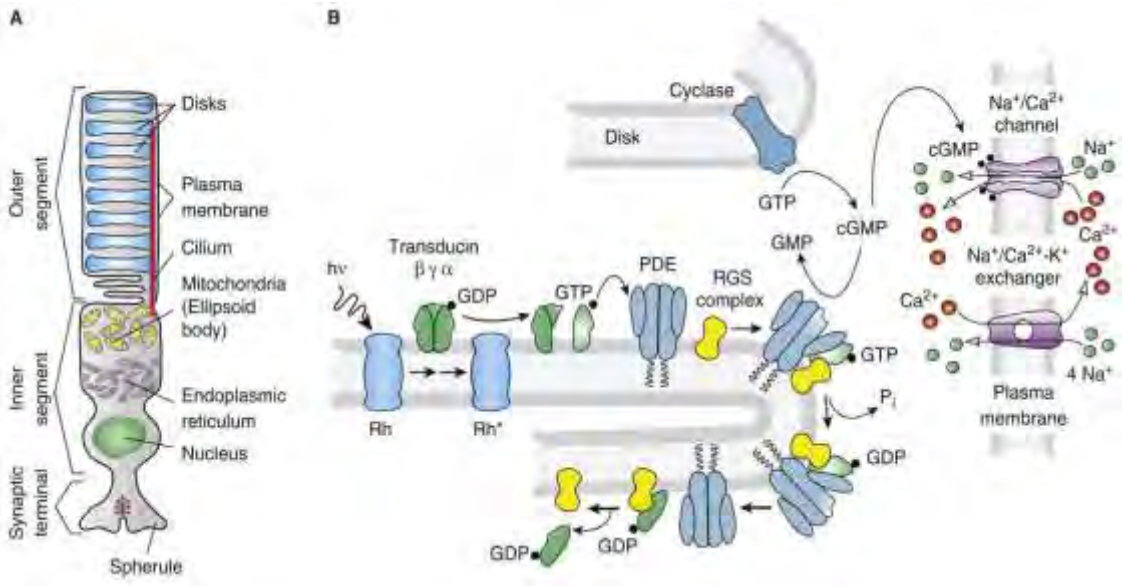


Fig 1.8. Mechanism of Phototransduction (Fain et al., 2010)**.**

Table 1.2: Mutation identified in Pakistan in different genes associated with RP.

Gene	Nucleotide Variant	Protein Variant	Phenotype	Families	Patients	References
ABCA4	c.6658C>T	p. (Gln2220*)	arRP	1	6	(Khan et al., 2021)
BEST1	c.418C>G	p. (Leu140)	arRP	1	4	(Haque et al., 2022)
CERKL	c.316C>A	p. (Arg106Ser)	arRP	1	3	(Ali et al., 2008)
CERKL	c.847C>T	p. (Arg283*)	arRP	1	6	(Kannabiran et

						al., 2012)
CLRN 1	c.92C>T	p. (pro31Leu)	arRP	1	6	(Ali et al., 2008)
CNGA 1	c.626-627del	p. (Ile209Serfs*26)	arRP	1	7	(Zhang et al., 2004)
CNGA 1	c.1298G>A	p. (Gly433Asp)	arRP	1	3	(Khan et al., 2014)
CNGB 1	c.2284C>T	p. (Arg762Cys)	arRP	1	5	(Haque et al., 2022)
CRB1	c.2536G>A	P. (Gly846Arg)	arRP	1	6	(Ilyas et al., 2020)
CRB1	c.3101T>C	p. (Leu989Thr)	arRP	1	4	(Sultan et al., 2018)
CRB1	c.3347T>C	p. (Leu1071Pro)	arRP	1	7	(Zobor et al., 2022)
CRB1	c.3343-3352del	p. (Gly1115Ilefs*23)	arRP	1	9	(Ali et al., 2008)
CRB1	c.2234C>T	p. (Thr745Met)	arRP	1	2	(Haque et al., 2022)
DHX3 8	c.995G>A	p. (Gly332Asp)	arRP	1	4	(Wang et al., 2019)
IMPG 2	c.1680T>A	p. (Tyr560*)	arRP	1	2	(Yusuf et al., 2019)
PDE6 A	c.304C>A	p. (Arg102Ser)	arRP	2	5	(Khan et al., 2021)
PDE6 A	c.889C>T	p. (Gly297Ser)	arRP	1	4	(Qures

						hi & Steel, 2020)
PDE6A	c.1264-2A>G	p. (?)	arRP	1	5	(Hayashi et al., 2021)
PDE6A	c.2218-2219insT	p. (Ala740Valfs*2)	arRP	1	3	(Wang et al., 2019)
PDE6B	c.1160C>T	p. (Pro387Leu)	arRP	1	6	(Zafar et al., 2017)
PDE6B	c.1655G>A	p. (Arg552Gln)	arRP	1	9	(Yusuf et al., 2019)
PDE6B	c.1722+1G>A	p. (?)	arRP	1	4	(Newton & Megaw, 2020)
PROM1	c.1726C>T	p. (Gln576*)	arRP	1	6	(Sultan et al., 2018)
RHO	c.448G>A	p. (Glu150Lys)	arRP	2	6	(Wheway et al., 2020)
RP1	c.1458-1461dup	p. (Glu488*)	arRP	2	9	(Haque et al., 2022)
RP1	c.4555del	p. (Arg1519Glufs*2)	arRP	1	5	(Zobor et al., 2022)
RP1	c.5252del	p. (Asn1751Ilefs*4)	arRP	1	4	(Hayashi et al., 2021)
SPATA7	c.253C>T	p. (Arg85*)	arRP/arLCA	2	3	(Takahashi et al., 2018)

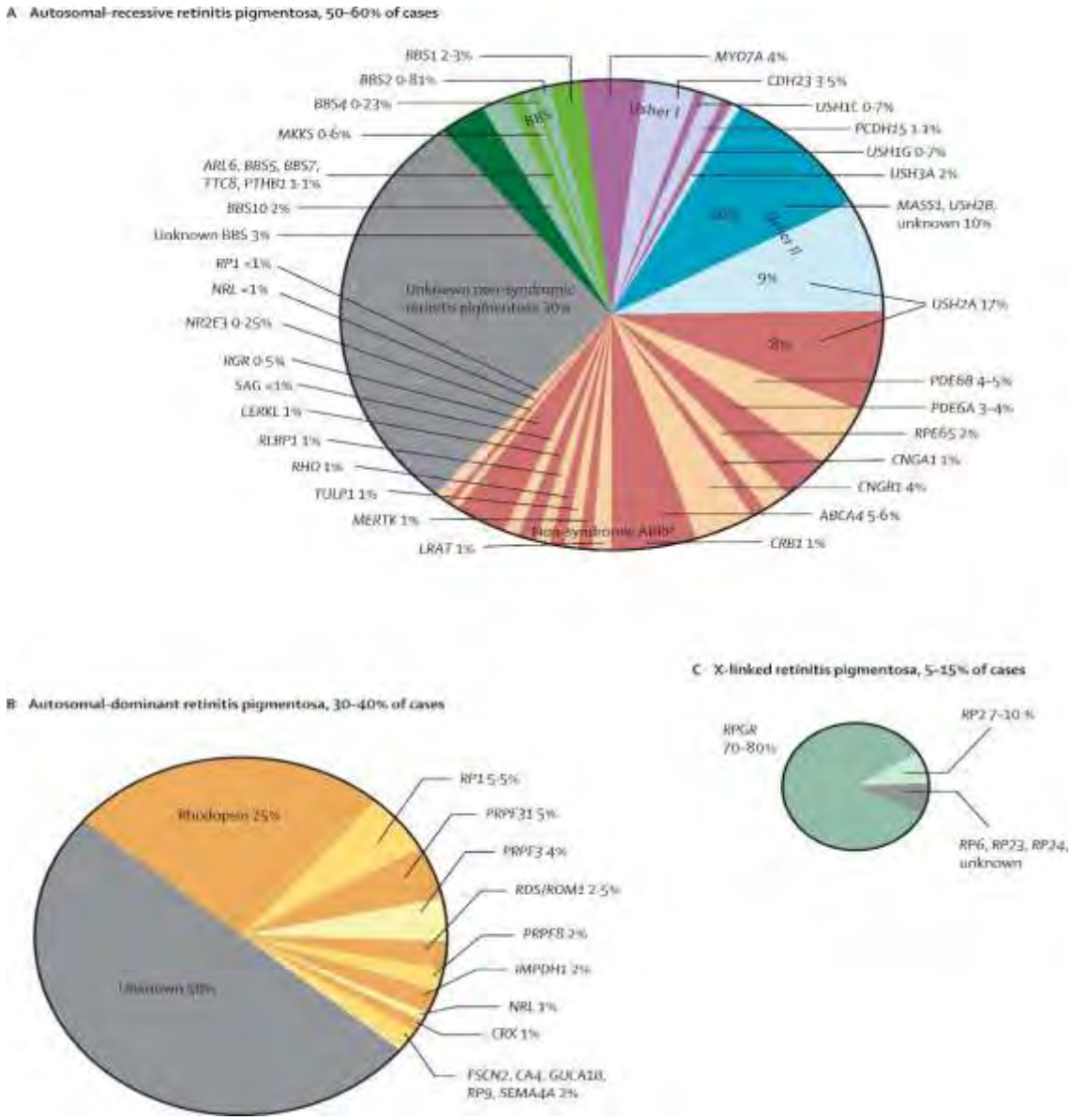


Fig 1.9: Genes and their relative contribution to retinitis pigmentosa (Hartong et al., 2006)**.**

1.5. Objectives:

Objectives of the current study are:

- To enroll clinically diagnosed Retinitis pigmentosa cases having positive family history.
- To screen prevalent mutation of PDE6A gene in all enrolled cases of non-syndromic RP.
- To find relative incidence of Syndromic and Non-Syndromic Retinitis Pigmentosa.

2. MATERIALS AND METHODS

2.1 Ethical Approval:

The current study was approved by the Bio-Ethical committee, Faculty of Biological Sciences, Quaid-e-Azam University Islamabad, Pakistan and Al-Shifa Trust Eye Hospital Rawalpindi, Pakistan.

2.2 Identification of families:

Families having Retinitis Pigmentosa (RP) affected patients were identified based on clinical diagnosis. The patients of Retinitis Pigmentosa (RP) and their 1st degree relatives who visited Al-Shifa trust eye hospital, Rawalpindi during August 2022 to February 2023 are included in this study.

2.3 Family Pedigree:

To determine the genetic relationship among the individuals of the family, a detailed interview was conducted from the normal elder person of the family. The mode of inheritance was determined from the pedigree analysis. HaploPainter 1.043 was used to draw the pedigree of each family. In the drawn pedigrees the square represent male while circle represent female. The hollow symbols represent the normal individuals while the filled symbols represent Retinitis Pigmentosa Patients. Th diagonal line on the symbols represents the deceased individuals. The double lines between the two symbols show the cousin marriage. To represent different generations, Roman numerals were used while the individuals in each generation were denoted by Arabic numerals.

2.4 Blood Collection:

Sampling from the families from different regions of Pakistan was carried out at Al-Shifa Trust Eye Hospital Rawalpindi, Punjab. All the patients included in the study voluntarily participated in sampling and were clinically diagnosed with Retinitis Pigmentosa (RP) by the physician according to the WHO criteria.

2.5 Exclusion Criteria:

- All the patients diagnosed with Retinitis pigmentosa with no family history were excluded.

- Furthermore, patients with any other eye disease other than RP were also excluded.
- Patients full filling inclusion criteria but not willing to participate were also excluded.

2.6 Inclusion Criteria:

All clinically diagnosed Retinitis Pigmentosa Patients having positive family history of disease and willing to participate in this study were included.

2.7 Samples Collection:

5 ml blood was taken from all the participants in properly labelled EDTA vacutainer tubes with written consent. Label included the individual's name, parent name and Unique Anonymous Identification (UAI) number assigned to each individual accordingly to keep them differentiated from each other. To prevent blood from clotting, EDTA contained in tubes was mixed with blood by shaking the tubes. Then the blood was stored at -4°C in the refrigerator (PEL).

2.8 Extraction of Genomic DNA:

The DNA extraction was performed by two different methods.

A. Phenol Chloroform method:

1st Day of DNA Extraction

- EDTA coated tubes having blood samples were placed at room temperature for 10-15 minutes that allowed the tubes to carry out Red Blood Cells (RBCs) lysis.
- The blood was transferred to the Falcon tubes of 50 ml labelled with individual UAI number.
- Washing buffer was prepared by adding 10 ml 1M Tris-HCl, 4 ml 2nM EDTA in a 1000 ml bottle and raised the final volume up to the brim of bottle which is 1000 ml with autoclaved distilled water and mixed it well.
- Falcon tubes containing 5 ml blood were filled with washing buffer up to 40 ml.
- In order to mix it well, the Falcon tubes were vortexed and then kept at room temperature for 10-15 minutes.

- The Falcon tubes were centrifuged for 20-35 minutes at 20°C and 3000 rpm.
- For further procedure, 20 ml supernatant was discarded with the help of autoclaved glass pipettes and white blood cells (WBCs) pellet was retained at the bottom of Falcon tubes.
- The pellet was broken by using vortex or tapping it.
- Falcon tubes having blood were refilled with washing buffer up to 40 ml and mixed well by using vortex or tapping it.
- The Falcon tubes were centrifuged for 20 minutes at 20°C and 3000 rpm.
- 20 ml supernatant was discarded with the help of autoclaved glass pipettes and white blood cells (WBCs) pellet was retained at the bottom of Falcon tubes.
- Step viii, ix, x, xi were repeated until light pink or clear WBCs pellet was obtained, in each repeat the amount of supernatant to be discarded was increased.
- All the supernatant was discarded leaving just pellet behind.
- The broken clear pellet was dissolved in 5 µl of 20% Sodium Dodecyl Sulphate (SDS), 3 ml of Tris-NaCl-EDTA (TNE) Buffer and 25 µl of Proteinase Kinase (PK) (10 µg/µl) according to the initial volume of blood i.e., 5 ml, used for DNA extraction.
- After vortex, the falcon tubes were incubated at 37°C for overnight.

2nd Day of DNA Extraction:

- Falcon tubes were taken out of the incubator, digestion of pellet was confirmed and were placed at room temperature. In case of presence of undigested, additional amount of protein kinase was added into the falcon tubes, vortex was used to digest pellet and the falcon tubes were placed in incubator again for 2 to 3 hours at 37°C.
- 500 µl of 6M Sodium Chloride (NaCl) was added in each tube. After vigorous shaking, the tubes were placed in ice for 10 to 15 minutes.
- PCI mixture was prepared which contain Phenol, Chloroform and Isoamyl Alcohol having ratio of 25:24:1 respectively. 1 ml of PCI mixture was added in each tube and gently inverted the tube to mix it well.
- Tubes were centrifuged for 20 minutes at 20°C and 3000 rpm.

- After centrifugation, three layers were visualized. The upper layers contain DNA, the middle whitish layer contains proteins and the lower layer contain PCI solution.
- Carefully transferred the upper layer which contain the DNA, into new pre-labeled falcon tubes by using micropipette to avoid contamination.
- 5 ml of isopropanol was added in each tube and mixed by gently inverting tubes until DNA threads become visible by precipitation.
- Falcon tubes were left for 5 to 10 minutes at room temperature.
- Tubes were centrifuged for 20 minutes at 20°C and 3000 rpm and DNA pallet was formed.
- After centrifuge, supernatant was discarded very carefully, leaving the DNA pellet adhered to the walls of tubes.
- DNA pallets were washed with ethanol by adding 5 ml of chilled 70% Ethanol.
- Tubes were centrifuged for 20 minutes at 20°C and 3000 rpm.
- After centrifuge, supernatant was discarded very carefully, leaving the DNA pallet adhered to the walls of tubes.
- The DNA pallet was dried by keeping the falcon tubes inverted on tissue paper for 2 to 3 hours.
- The pallet was dissolved in TE Buffer (according to the quantity of DNA threads), vortexed and placed in incubator for overnight at 37°C to dissolve the DNA in buffer.

3rd Day of DNA Extraction:

- Caps of Falcon tubes were sealed with parafilm strips.
- The tubes were placed for heat shock in water bath at 70°C for 1 hour. Heat shock inactivates the nucleases and prevent DNA from denaturation.
- Tubes were placed at room temperature for 5 minutes.
- Short spin was given to the tubes by centrifuge to mix the DNA and buffer properly.
- DNA solutions were transferred to pre-labelled and autoclaved screw cap tubes.
- The screw cap tubes containing DNA sample were stored at -20°C in properly labelled cryobox.

Table 2.1: Concentration and Composition of solutions used for DNA extraction.

Solution	Concentration and Chemical Composition
TE Buffer	2mM EDTA, 10 mM Tris HCl (PH= 8.0)
TNE Buffer	10 mM Tris HCl, 400mM NaCl, 2mM EDTA
SDS	20%
Proteinase Kinase	10mg/ml
NaCl	6M
Ethanol	70%
Chloroform: Isoamyl Alcohol	24:1
PCI (Phenol-Chloroform: Isoamyl Alcohol) Solution	P (50): C (48): I (2)
Isopropanol	100%

B. WizPrep gDNA Kit Method

DNA is extracted from whole blood simply and more conveniently, in a short period of time, that is from half to a few hours, depending on sample size. There is fast and easy processing using a rapid spin-column format, with no use of phenol or chloroform. The protocol is given below.

- Take 200µl of whole blood, add 200 µl of GB Buffer and 20 µl of Proteinase K.
- Vortex the above mixture and incubate at 56°C for 20 minutes.
- Add 200µl of 100% Ethanol in above mixture after incubation.
- Vortex the tubes to mix properly.
- Transfer the lysate (above mixture) into column that is attached with a collecting tube.
- Give short spin to the column (1 minute at 13000 rpm).
- Discard the filtrate and transfer the column to new collection tube.
- Add 500µl of W1 Buffer in column and centrifuge for 1 minute at 13000 rpm.
- Discard the filtrate, add 500µl of W2 Buffer, and centrifuge it for 1 minute at 13000 rpm.

- Discard filtrate and shift the column to newly labelled Eppendorf tubes.
- Add 50-100 µl Elution Buffer and keep it at room temperature for 1 minute.
- Centrifuge it for 1 minute at 13000 rpm and discard the column.
- The eluted purified DNA is extracted.
- Store the extracted DNA at 20°C for a few days and at -70°C for long term storage.

2.9 Agarose Gel Electrophoresis (1%):

- For the confirmation of extracted DNA sample, gel electrophoresis was done. The protocol of Agarose Gel Electrophoresis (1%) is given below.
- 1% agarose gel was prepared by dissolving 0.5 gm of agarose powder in 50 mL of 1X TBE (Tris-Boric Acid- EDTA) buffer in a conical flask.
- 1X TBE buffer was prepared by adding 900 ml distilled water in 100 ml of 10X TBE in a bottle of 1000 ml as represented in Table 2.2. While 10X TBE buffer was prepared by adding 40 ml 0.5M EDTA, 108 gm Tris, 54 gm Boric acid and raised the final volume to 1000 ml by adding distilled water in it and adjusting the pH at 8.0.
- To make a clear solution, the mixture was placed in microwave for 1 to 2 minutes. Before placing the flask in microwave oven, the flask's opening was covered with Aluminum foil.
  - Then it is allowed to cool at room temperature for few 1-2 minutes.
  - 5 µl Ethidium Bromide (EtBr) was added in the gel mixture and gently shaken for mixing the components. EtBr is an intercalating agent and is used for DNA identification under Ultraviolet (U.V) light.
  - Casting tray and comb was placed in a rack.
  - The clear solution was poured in the casting tray such that there was no bubble formation in the casting tray.
  - Allowed it to solidify (Polymerization) for 30 to 40 minutes at room temperature.
  - After solidification of gel, the comb was gently removed, and the gel was placed in the gel tank (Cleaver Scientific Limited, CS-3000V) which was filled with the running buffer i.e., 1X TBE buffer.
  - The loading samples were placed by mixing 3 µl extracted DNA of each sample with 3 µl of 6X Bromophenol blue (Loading dye).
  - The gel electrophoresis apparatus was run at 120 Volts for 25 minutes.

- After completion of running, the gel was visualized under UV by using Gel Documentation System (Cleaver Scientific Limited).

Table 2.2: Composition of agarose gel and other required Chemicals

S. No.	Solutions	Composition
1.	**1% Agarose Gel (50 ml)**	1X TBE (50 ml) Agarose (0.5 g) Ethidium Bromide (2 µl)
2.	**2% Agarose Gel (50 ml)**	10X TBE (5 ml) Agarose (1.0 g) Ethidium Bromide (5 µl) Distilled water (45 ml)
3.	**Gel preparation buffer (10X TBE)**	Boric Acid (27.5 g) EDTA (3.65 g) Tris (54 g) Deionized water (500 ml)
4.	**Gel Running Buffer (1X TBE)**	10X TBE (1 part) Distilled Water (9 part)
5.	**Ethidium Bromide (50 ml)**	Auto- calved filtered water (25 ml) Ethidium Bromide (0.5 g)
6.	**Loading Dye**	Auto- calved filtered water (25 ml) Bromophenol blue (0.0875) Sucrose (10 g)

2.10 Nanodrop:

To find the concentration and purity of the extracted DNA, the DNA samples were quantified (concentration) and qualified (purity) on nanodrop (Thermo-Scientific 2000) using T.E buffer as a blank.

2.11 FASTA Sequence of Selected Exon and Mutation:

GTGTCAGATATTTAAGAAAACTAACAGAGGTCAGAGAAGACACACCTACA
GCAAGTAGACTGTCCCTGTGCTGCCTTTTTGCAACCCCTGCTTTGGCAGTG
CTCAAGCCCACCTCCTGCTCTGTGCAGACATCTCTTCTTTGCTCTTACTAGA

CCAAGGTGAAAGAAAACTCTCACCTTCTCCCATCTGGCCCCACAGCATCTG
GAACACACTGATCCTCATAATCCTTGTTCTTGAGAAATATTAATGACTTAATC
TCCCAAGCTTGCTCCCTCTCCTGTGCAGGCCATCTCAGTATGTTTTGCAGAC
AAGACCCAGAGAAGTCCAGACTGGACTTGTTGCAGACTGCAAAACTGCCA
TTGGAAGGCCTCCGTCCCAGTCCTTCTACAGAGTAGCCAGTGGGATTCCCA
GCCATGGGCGAGGTGACAGCAGAGGAGGTGGAGAAGTTCCTGGACTCGA
ATATTGGCTTTGCCAAACAGTACTACAACCTCCACTACCGGGCCAAGCTCA
TCTCCGACCTCCTTGGGGCCAAGGAGGCTGCCGTGGACTTCAGCAACTAC
CACTCCCCGAGCAGCATGGAGGAGAGCGAAATCATCTTTGATCTCCTGCGG
GACTTTCAGGAGAATTTACAGACAGAGAAATGCATCTTCAATGTCATGAAG
AAGCTGTGCTTCCTCCTGCAGGCAGACCGCATGAGCCTGTTCATGTACCGG
ACCCGCAATGGCATCGCAGAGCTGGCCACCAGGCTTTTCAATGTCCACAA
GGATGCTGTCCTCGAGGACTGCCTGGTGATGCCCGACCAAGAGATCGTCTT
CCCTTTGGACATGGGCATCGTGGGCCATGTCGCACACTCTAAGAAGATTGC
TAACGTCCCCAACACAGAGGAGGTACTCTCTTCCCCATGAGAGAGAGGGC
ATGGGGCATTATTACATGGAGTTCTGGGGTACAGGTGGGGTGAGGGGCATT
GGCCACCAAGACAAGGCTGGTGACAACATGGTGCTTCTTCTTTATTTATTTA
CATATTTACTCATAATTCTTTATTTTGAAAAAAAAATTCAAACTTAACAGAA
AAGTTTGAAATAATACAGAGAACTGTATGTCTTTCATCCAAATTTATCATTC
ATTAACATCTT

2.12. Primer Designing:

For this study, primers were designed using primer-3 software (https://primer3.ut.ee/) for the amplification of the specified region of PDE6A gene containing PDE6α c.304C>A, p.R102S mutation. All the conditions such as annealing temperature of primers, amplicon size, salt concentration and primer length for the required primers were adjusted at optimum level. Reference sequence was obtained from the Ensemble website (https://asia.ensembl.org/Homo_sapiens/Info/Index) that was used to design required primers. Blast like alignment tool (BLAT) on UCSC genome browser (https://genome.ucsc.edu/cgi-bin/hgBlat) was used to confirm the specificity f the selected primers. In addition, In-silico PCR tool on UCSC genome browser (https://genome.ucsc.edu/cgi-bin/hgPcr) was used to verify the amplicon size for the primers. Locus of mutation, primer sequence, product size and melting temperature for each primer is mentioned in table 2.3.The primer used was shown in Table 2.3.

Table 2.3. Primers for Selected Exon

Gene	Exon	Primer	Sequence (5'→ 3')	Primer Length	Product Size(bp)	T_M
PDE6A	2	Forward primer	5'-ACTACCGGGCCAAGCTCATCTCCGACCT-3'	28	367	55° C
		Reverse primer	5'-TCGTGGGCCATGTCGCACACTCTAAGAA-3'	28		

2.13. Primer Dilution:

The initial concentration of the ordered primer was 50 picomole/µl. Further dilution of primers was done to make final concentration of 10 picomole/µl.

2.14. Polymerase Chain Reaction:

The selected exon (Exon 2) was amplified by using polymerase chain reaction (PCR) in all the members of 4 randomly selected families.PCR was performed in PCR tubes (Axygen, USA) having 200µl capacity. The chemicals (Thermo-Scientific PCR Kit) and their volume used in this reaction mixture are given in table 2.4.

Table 2.4. Chemicals used in PCR Mixture

Sr.No.	Chemical	Concentration	Volume (µl) for single PCR Reaction
1	Taq. Buffer $[(NH_4)_2SO_4]$	10X	2.5µl
2	dNTPs	2.5mM	2.5µl
3	$MgCl_2$	25mM	2µl
4	Forward Primer	10pmol/µl	0.5µl
5	Reverse Primer	10pmol/µl	0.5µl
6	DNA	>100ng/µl	2µl
7	Taq Polymerase	5U/µl	0.5µl
8	PCR Water		14.5µl
	Total Volume		**25µl**

The PCR tubes were subjected for short spin in microfuge at 3000 rpm for 1 minute to mix the contents before placing them in thermocycler (Bio-Rad T100) for PCR reaction are given in table 2.5.

Table 2.5. Conditions for PCR Cycles

Step	Temperature	Time	Cycle
Initial Denaturation	96°C	5 min	1X
Denaturation	95°C	45 sec	40X
Annealing	55°C	45 sec	
Extension	72°C	60 sec	
Final Extension	72°C	10 min	1X
Hold	25°C	∞	

2.15. PCR Product Confirmation:

To confirm the PCR products, 2% agarose gel was prepared by adding 1.6g of agarose powder in 80ml of 1X TAE buffer along with 2µl Ethidium Bromide. 3-4µl of each sample i.e., PCR product was mixed with 2µl of 6X florescent dye (Bromophenol blue). DNA samples were allowed to run for 40 min at 120V against 2% Agarose gel along with ladder of 1kb. Then the gel was visualized on Gel Documentation System (Cleaver Scientific Limited) to confirm the amplification of targeted segment of PDE6A Gene.

2.16. PCR Product Purification:

For purification of PCR product, a purification kit (Thermo-Scientific) was used. The protocol followed for PCR product purification is given below.

- An equal volume of binding buffer was added to PCR product.
- After tapping the tubes, the mixture was left for 1 minute at room temperature.
- Tubes were centrifuged at 13000 rpm for 2 minutes to mix it well.
- Samples were transferred to pre-labelled spin column tubes with collection tube attached.
- Spin column tubes were centrifuged at 13000 rpm for 1 minute.
- 350µl washing buffer was added in each sample and again centrifuged at 13000 rpm for 1 minute.
- Samples were kept at room temperature for 2 minutes.
- 25µl Elution buffer was added in each sample. Elution buffer was kept in incubator at 70°C before use.
- The spin column tubes were placed in pre-labelled Eppendorf tubes.
- The samples were kept at room temperature for 2 minutes.
- Tubes were centrifuged at 13000 rpm for 1 minute.
- Samples were run on 2% Agarose Gel to check the purity of PCR products.

2.17. Sequencing:

Sanger's sequencing was performed for each sample to identify the sequence of nucleotides. All the samples were sent for Commercial Sanger's sequencing (Macrogen, South Korea).

2.18. Mutation Analysis:

The sequenced data was aligned against the reference sequence taken from Ensemblegenomebrowser(https://asia.ensembl.org/Homo_sapiens/Info/Index). The sequences were put into Bio-edit (v.7.2.0) and were aligned with reference sequence. Mutation tester was used to check the conflict. Furthermore, I-Mutant (https://folding.biofold.org/imutant/),PROVEAN(http://provean.jcvi.org/index.php) and gnomAD (https://gnomad.broadinstitute.org/) were also used for novel and previously reported variants.Uniport

(https://www.uniprot.org/uniprotkb?query=PDE6A) was consulted to check the variant consequences at protein level. Chromas 2.66 was used for chromatogram analysis. Moreover, other tools such as PROVEAN

(https://provean.jcvi.org/index.php), SIFT (https://sift.bii.a-star.edu.sg/) and ITASER (https://zhanggroup.org/I-TASSER/) were also used. HOPE (Have Your Protein Explained) tool (https://www3.cmbi.umcn.nl/hope/input/) was also used to know the effects of substituted amino acid on protein structure and biochemical nature.

3. RESULTS

3.1 Clinical Characteristics:

Blood samples of twenty families belonging to different regions of Pakistan were collected at Al-Shifa Eye Trust Hospital, Rawalpindi. All families have positive history for Retinitis Pigmentosa and at least two individuals were diagnosed with the RP. All the families have consanguineous marriages. Among 20 families, 4 are syndromic and 16 are non-syndromic RP families. Total individuals from which blood samples were collected were 111 including 55 males and 56 females. Among them, 55 individuals were control, and 56 individuals are diagnosed with RP including 32 males and 24 females. All the RP patients have progressive RP. The ratio of male RP patients was greater than the female RP patients i.e., 57.14% males and 42.86% females. Only four randomly chosen families are processed and sent for Commercial sequencing from Macrogen, South Korea. Only 4 families were selected due to limit of samples we can sequence.

From 4 families, proband was selected for sequence analysis of Exon 2 of PDE6A gene. The PDE6A gene was selected for this study it constitutes about 4-8% of human RP patients (Bayés et al., 1995; Dryja et al., 1999; Dvir et al., 2010). Many PDE6A mutations are known to cause RP but, so far, they have been relatively little studied (Sakamoto et al., 2009). Exon 2 of PDE6A have never been studied separately so this study was designed to genetically analyze the exon 2 of PDE6A gene. Till now only one silent polymorphism has been observed in exon 2 at c. 618G>A (Riazuddin et al., 2006) and one mutation p.arg102ser is found in exon 2 (Khan et al., 2021).

Initially, the families were recruited based on common RP symptoms like night blindness and visual impairment. The visual acuity was variable among all probands. Furthermore, the RP was diagnosed by the ophthalmologist based on fundus observation including bony spicules, attenuated arteries, pigmentation, and waxy pallor disc as shown in fig.3.1.

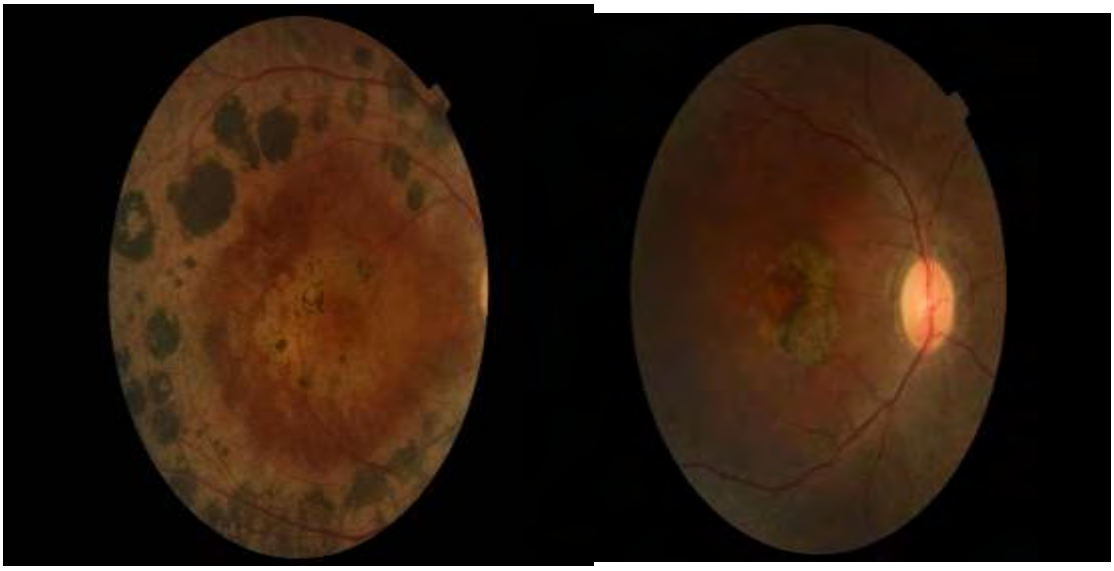
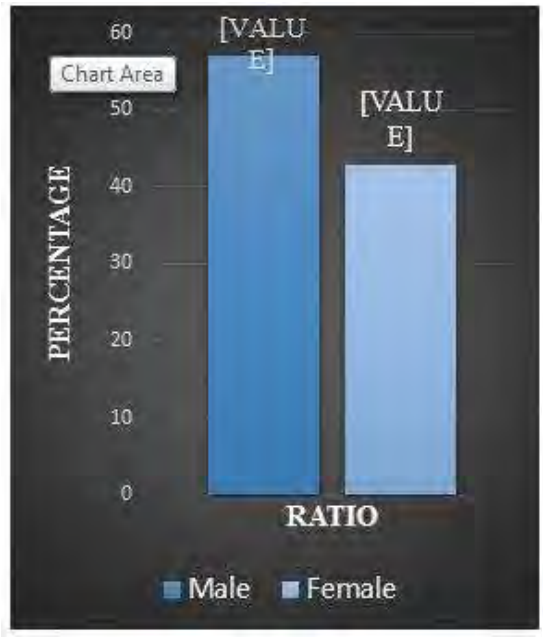
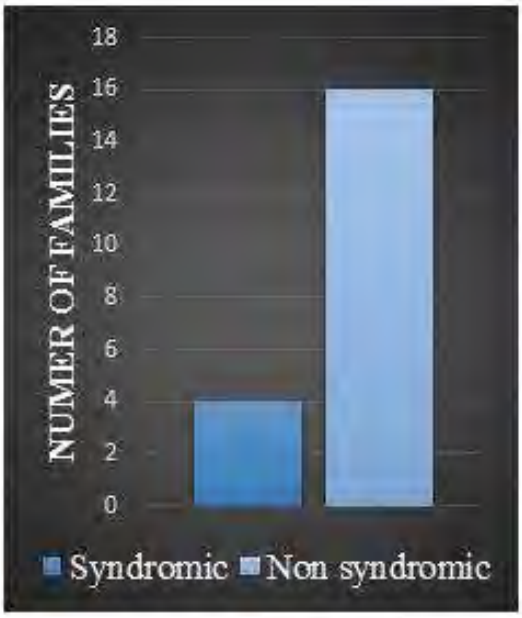
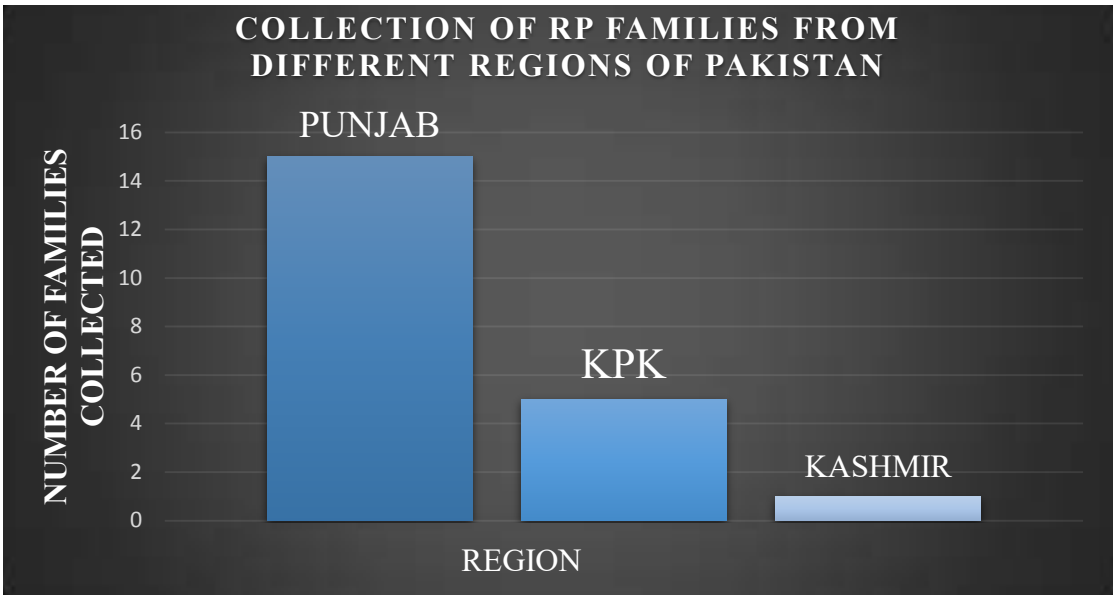


Fig. 3.1: Representative fundus photographs of RP Patients





(a) **(b)**



(c)

Fig. 3.2: (a) Graph representing the percentage of male and female affected individuals in all collected RP families. (b) Graph representing the number of collected Syndromic and Non-Syndromic families. (c) Graph representing number of RP families from different regions of Pakistan.

Detailed description of four selected non-syndromic RP families:

3.1.1. Family RP-58:

The family RP-58 belongs to District Gujrat, Punjab, Pakistan. This family consisted of 33 members having three affected members, all are female (IV-II, IV-III, and IV-X). The descriptive data was collected from all alive members. The age of affected individuals at the time of enrolment were 20 years, and 17 years respectively. The third individual was not available. All the affected members of the family had progressive night blindness, visual impairment, and bilateral RP. This family had Non-Syndromic RP. The pedigree shows the pattern of RP as autosomal recessive. Figure 3.3(A) represents the pedigree drawing of family that is important to ascertain mode of inheritance and family history of RP phenotype as well as relationship of other members with proband.

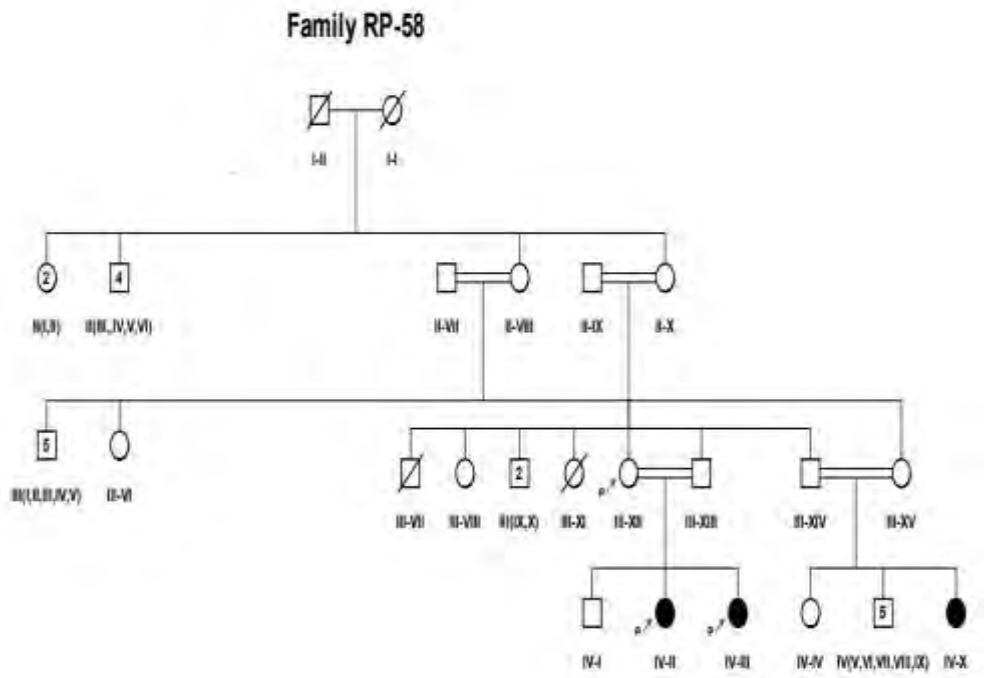


Fig.3.3 (A) Pedigree of RP-58 Family

3.1.2. Family RP-59:

The family RP-59 belongs to District Mandi Bahauddin, Punjab, Pakistan. This family consisted of 21 members having two affected members and they are male (V-I, and V-II). The descriptive data was collected from all alive members. The age of affected individuals at the time of enrolment were 12 years, and 10 years respectively. All the affected members of the family had progressive night blindness, visual impairment, and bilateral RP. This family had Non-Syndromic RP. The pedigree shows the pattern of RP as autosomal recessive. Figure 3.3(B) represents the pedigree drawing of family that is important to ascertain mode of inheritance and family history of RP phenotype as well as relationship of other members with proband.

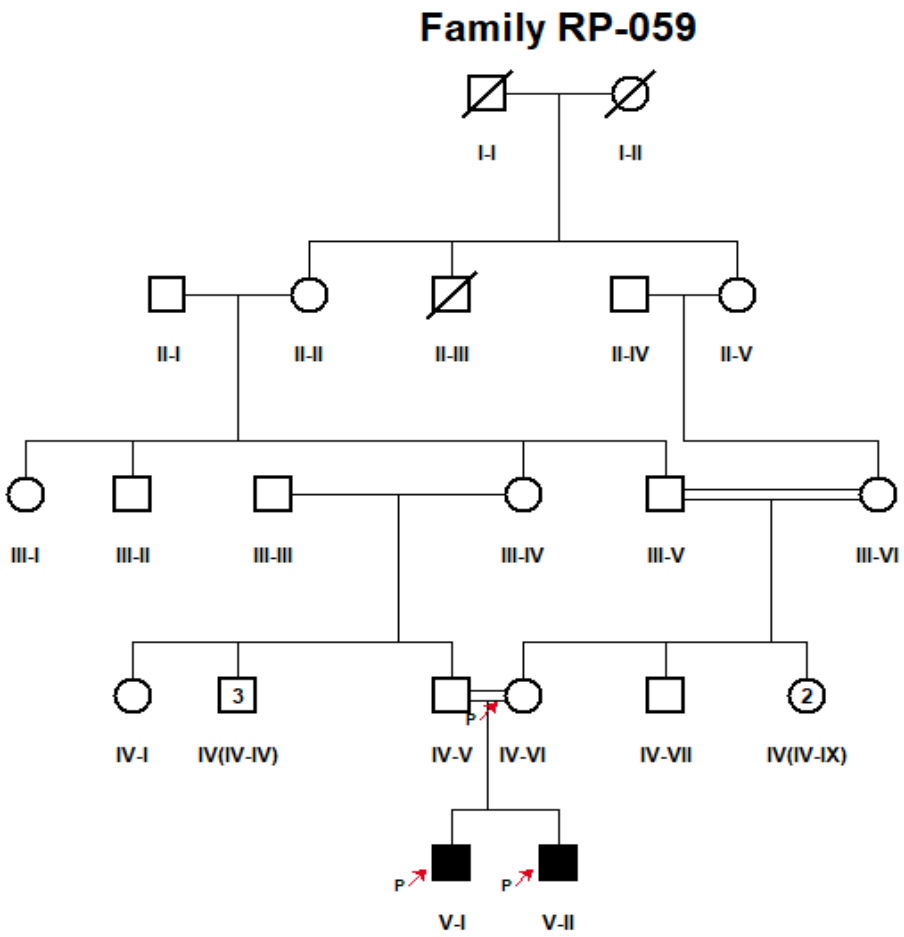


Fig.3.3 (B) Pedigree of RP-58 Family.

3.1.3. Family RP-60:

The family RP-60 belongs to District Sudhanoti, Kashmir, Pakistan. This family consisted of 12 members having three affected members and two were female (IV-II, and IV-III) and one was male (IV-I). The descriptive data was collected from all alive members. The age of affected individuals at the time of enrolment were 23 years, 21 years, and 25 years respectively. All the affected members of the family had progressive night blindness, visual impairment, and bilateral RP. This family had Non-Syndromic RP. The pedigree shows the pattern of RP as autosomal recessive. Figure 3.3(C) represents the the pedigree drawing of family that is important to ascertain mode of inheritance and family history of RP phenotype as well as relationship of other members with proband.

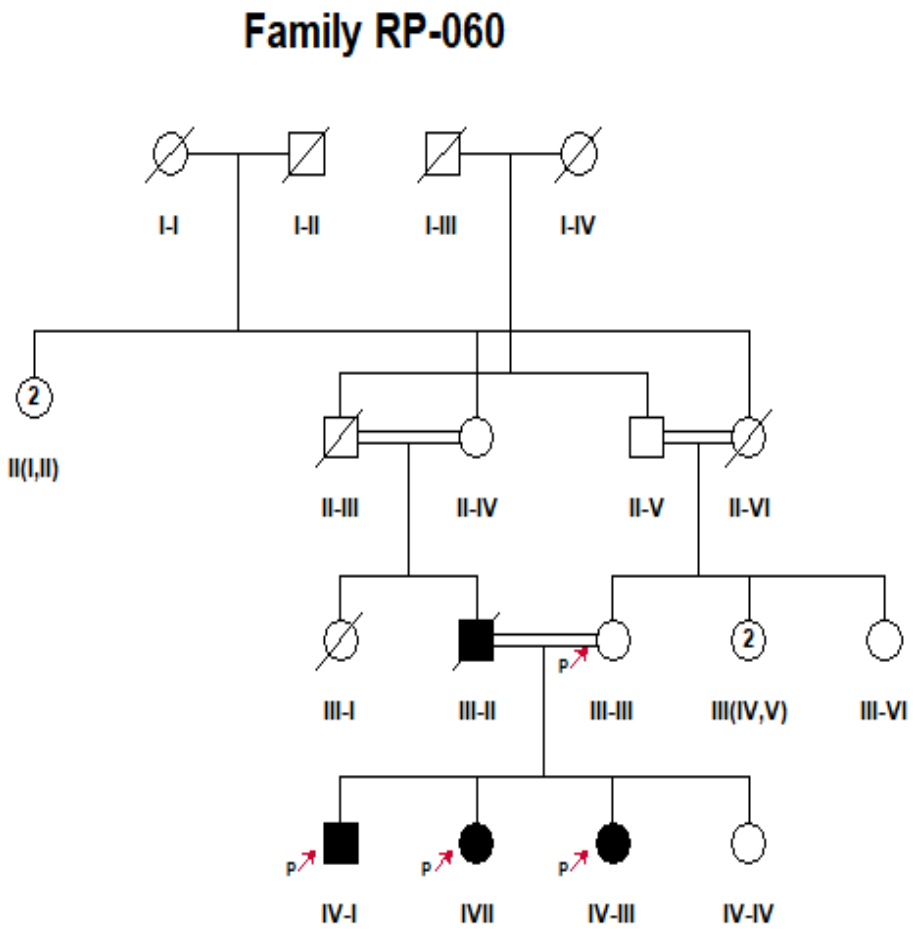


Fig. 3.3 (C) Pedigree of RP-60 Family.

3.1.4. Family RP-99:

The family RP-99 belongs to District Mirpur, Punjab, Pakistan. This family consisted of 28 members having two affected members and one was female (IV-IV) and one was male (IV-V). The descriptive data was collected from all alive members. The age of affected individuals at the time of enrolment were 20 years, and 23 years, respectively. All the affected members of the family had progressive night blindness, visual impairment, and bilateral RP. This family had Non-Syndromic RP. The pedigree shows the pattern of RP as autosomal recessive. Figure 3.3(D) represents the pedigree drawing of family that is important to ascertain mode of inheritance and family history of RP phenotype as well as relationship of other members with proband.

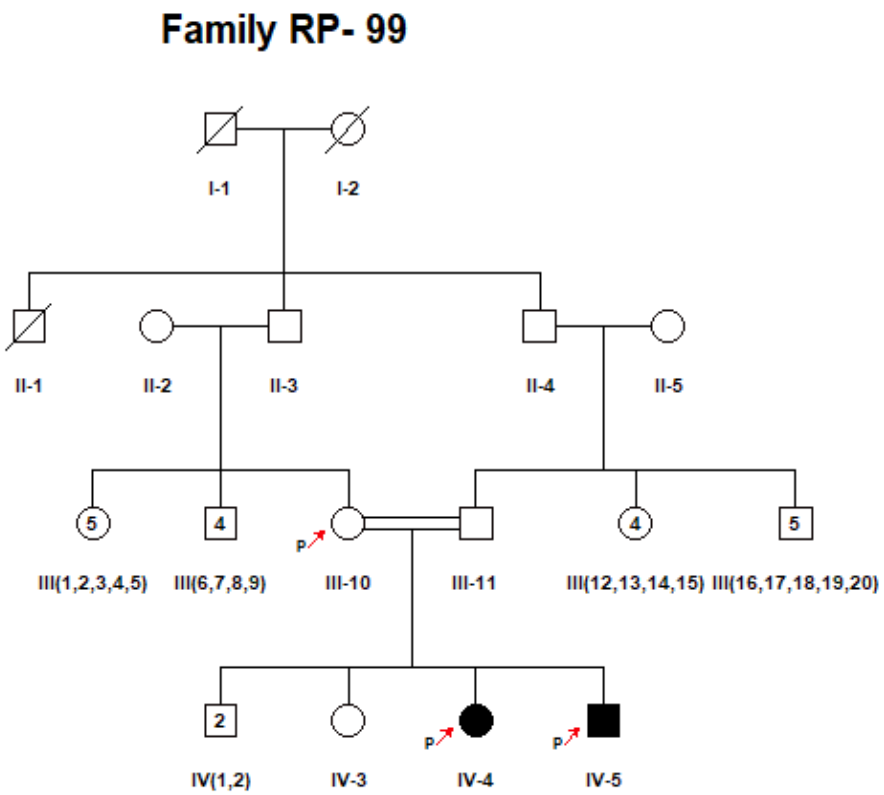


Fig.3.3 (D) Pedigree of RP-99 Family.

3.2. DNA Isolation:

The DNA was isolated from each blood sample and the mean concentration of the isolated DNA was up to 50ng/ with a purity value of 1.8 for each isolated sample as shown in fig. 3.4.

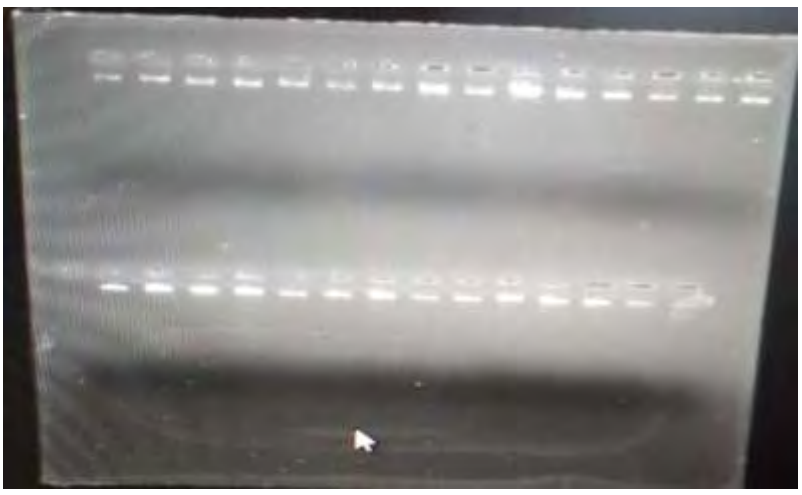


Fig.3.4. DNA isolated from the blood samples of RP families.

3.3. Primer Optimization:

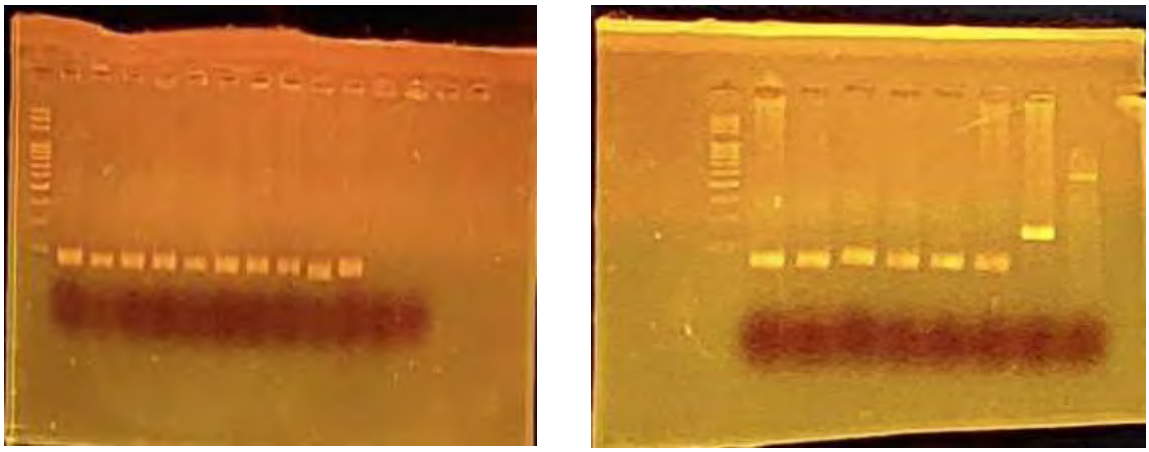
For optimization of primers gradient PCR was used. The annealing temperature, 55℃ were found for primer pair designed for mutation analysis of PDE6A gene.

3.4. Polymerase Chain Reaction:

Polymerase Chain Reaction (PCR) was performed to amplify the DNA samples of all the probands of chosen 4 families. The PCR products for polymorphism analysis Exon 2 of PDE6A gene were obtained by using PCR gradient. The confirmation of amplicon was done by running 2% of agarose gel electrophoresis and was observed in gel documentation system (Cleaver Scientific Limited).

3.5. PCR Product Purification:

For purification of PCR products, a purification kit (Thermo-Scientific) was used. All the PCR products of probands were purified and 20µl purified PCR products were obtained. The confirmation of amplicon was done by running 2% of agarose gel electrophoresis and was observed in gel documentation system. PCR amplicon of a family is shown in fig. 3.5 (a and b).



(a) **(b)**

Fig. 3.5 (a and b): Confirmation of purified PCR Products.

3.6. Sanger's Sequencing:

All the purified samples were sent for Commercial Sanger's sequencing (Macrogen, South Korea). Sequence reactions were carried out using big dye terminator chemistry. Capillary electrophoresis was used to separate the labelled DNA fragment, and a spectrum analyzer was used to confirm their detection. Each nucleotide (A, T, G,C) was labelled with a specific color for documentation. For Sanger's sequencing results visualization, Sequencher 5.4.6 and Chromas 2.6.6 was employed.

3.7. Genetic Analysis:

Mutations in PDE6A gene are known to cause RP in different populations globally. Exon 2 of PDE6A gene (Ensemble Transcript I.D: ENSG00000132915) was selected to screen polymorphism and mutation in selected families. PDE6A having Ensemble Transcript I.D: ENSG00000132915 was used as standard sequence alignment. All the 4 analyzed sequences did not show any polymorphism or mutation. Exon 2 is one of the 22 exons of the PDE6A gene. There is a high chance that there is no mutation in this exon. In total, more than 60 genes and 150 mutations are reported to be associated with RP worldwide (Kannabiran et al., 2022; Zafar et al., 2017). So, to understand underlying genetic cause of this disease we should also study other genes as well.

DISCUSSION

Retinitis Pigmentosa (RP) is characterized by the primary degeneration of the rod photoreceptors followed by the loss of cone photoreceptors (Verbakel et al., 2018). The characteristic feature of RP is the progressive loss of visual field. Three clinical findings typical of RP are vascular narrowing, presence of bone spicule pigmentation, and optic nerve pallor (Verbakel et al., 2018). The typical symptoms of RP include night blindness, progressive loss of peripheral vision, tunnel vision, and even blindness is observed in advanced stages of RP (Q. Gao et al., 2019). One of the macular conditions related to RP is cystoid macular edema (CME) causing the loss of central vision (Fahim et al., 1993). It has been reported that 50% of patients having RP also has CME, other macular complications such as macular hole and epiretinal membrane are also observed in RP patients (Strong et al., 2017).

Based on mode of inheritance pattern RP is categorized as sporadic, autosomal dominant (adRP), autosomal recessive (arRP), X- linked, mitochondrial and digenic (Kajiwara et al., 1994). If the disease is affecting only eyes, then it is known as "non-syndromic RP". When RP occurs in conjunction with other systemic diseases then it is referred as "syndromic RP" (Wolfrum & Nagel-Wolfrum, 2018). Milder symptoms appear in the RP patients whose family history indicate autosomal dominant mode of inheritance as compared to X-link inheritance whose patients show severe symptoms with loss of central vision (Mandal et al., 2023).

Around the globe, 285 million people are suffering from visual impairment. Amon them 2-3% are affected with Retinitis Pigmentosa (Colombo et al., 2021). This disease has a worldwide prevalence. More than 2.5 million people are affected by RP worldwide. Depending upon the demography of area, it's prevalence ranges from 1:4000 to 1:9000 (Toms et al., 2020;F.-J. Gao et al., 2019).

In this study, 20 cases are reported in which 80% (16 families) have non-syndromic RP while 20% cases (4 families) have syndromic RP. In previously reported data percentage of syndromic and non-syndromic RP is 15% and 85% respectively(Ferrari et al., 2011). In another study, the non-syndromic cases form 65-75% cases and syndromic cases form 25-35% of the total families collected (Menghini et al., 2020; Teo et al., 2021). 95% of families reported in this study has cousin marriages. It is reported that in Pakistan, the frequency of cousin marriages is 80% that contribute to

the high prevalence of autosomal recessive disorders (Ilyas et al., 2020). Studies shows that in Pakistan, about 95% of the retinal disorders are autosomal recessive due to frequent consanguinity among which RP is most common (Sultan et al., 2018).

In past decades, a rapid increase in cases of RP has been reported. In 2010, the number of RP cases reported in South Asia are, 58,528 cases in Pakistan, 58,963 cases in Bangladesh 391,570 cases in India, 15,482 cases in Afghanistan, 9318 cases in Sri Lanka, 1603 cases in Bhutan (Bouzidi et al., 2021;Sun et al., 2021;Thapa et al., 2020). In 2020, an increase in these numbers have been reported such that, 92,762 cases in Pakistan, 83,293 cases in Bangladesh, 588,320 cases in India, 21,821 cases in Afghanistan, 13,931 cases in Sri Lanka, 3887 cases in Bhutan (Karali et al., 2019;Van Cauwenbergh et al., 2017).

Autosomal recessive RP (arRP) is the most common form of retinal dystrophies in Pakistani population (Moore et al., 2020). Most common inheritance pattern is autosomal recessive in RP patients (Zafar et al., 2017). In this study 20% cases show autosomal dominant pattern of inheritance, 80% cases indicate autosomal recessive mode of inheritance, and no other inheritance pattern is observed. Studies on Pakistani population shows that 64% cases are of autosomal recessive retinitis pigmentosa, 10% cases are of autosomal recessive cone-rod dystrophy, 8% cases are of autosomal recessive congenital stationary night blindness, and 18% cases are of autosomal recessive Leber Congenital Amerosi (Maria et al., 2015;Shahzad et al., 2013). In another study 20-25% cases are of adRP and 15-20% are of arRP (Ferrari et al., 2011) yet in another study 20% cases are of arRP and 30% of adRP (O'Neal & Luther, 2022).

In total, more than 60 genes and 150 mutations are reported to be associated with RP worldwide. Most frequently reported genes associated with RP in Pakistani population are *SEMA4A, LCA5, RPGRIP1, RP1, TULIP1, AIPL1, PDE6B, PDE6A,* and *CRB1* (Kannabiran et al., 2022;Zafar et al., 2017).

The age of onset refers to the age at which the patient has reported the visual symptoms. The age of onset of RP, varies and depends upon its type (Verbakel et al., 2018;Verdina et al., 2021). RP Patients have no prominent symptoms in childhood; however, their vision is gradually lost during adolescence and adulthood because of progressive photoreceptor cell degeneration (Hartong et al., 2006). The average age of diagnosis of RP patients in this study was 22.64 years and the median was 20 years.

According to Tsujikawa et al., 2008, average age of diagnosis of patients with RP was 35.1 years, and the median was 36.5 years. The oldest age was 89 years, and the youngest age was 1 year (Tsujikawa et al., 2008), but in this study the youngest patient of RP was 3 years and the oldest age of RP patient was 89 years.

CONCLUSION

It has been determined that there is a high occurrence of the recessive form of Retinitis Pigmentosa in the community under study. *PDE6A* gene is one of many known RP genes that has a strong correlation with various types of RP. The *PDE6A* gene should first be checked for in all RP families. All impacted families should get genetic counselling, and cousin unions in RP-affected households should be strongly discouraged to reduce the incidence of the disease to subsequent generations.

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