Analysis of a common *PDE6A* Mutation (p.Arg102Ser) in Retinitis Pigmentosa Cases from Pakistani Population

By

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M.Phil. Molecular Biology

2020-2022

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A dissertation submitted in partial fulfillment of the requirement for the degree of Master of Philosophy

In

Molecular Biology

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2022


بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ
In the name of Allah, the most gracious, the most merciful

RESEARCH COMPLITION CERTIFICATE

Certified that this dissertation titled as **"Analysis of a common *PDE6A* Mutation (P.Arg102Ser) in Retinitis Pigmentosa Cases from Pakistani Population"** submitted by **Asfandyar Ahmad Khan**, 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 Molecular Biology.

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DECLARATION

I **Asfandyar Ahmad Khan,** student of **M.Phil. Molecular Biology**, Session **2020-2022**, hereby declare that the material and information contained in this thesis titled **"Analysis of a common *PDE6A* Mutation (P.Arg102Ser) in Retinitis Pigmentosa Cases from Pakistani Population"** is my own work and has not been printed, published or submitted as research work, thesis or publication in any University or Research Institute in Pakistan or abroad.

_____

Asfandyar Ahmad Khan

DEDICATION

This study is wholeheartedly dedicated

To

My Grand Parents

The pure, humble and gentle Souls

Whose love and prayers for me knew no bound.

My Beloved Parents

Who have been a constant source of inspiration for me and provided me moral, spiritual, and emotional support.

My Brother & Sisters

Who have always been there for me and encouraged me to complete this work.

My Supervisor

Dr. Sabika Firasat

Whose never-failing support, sympathy and encouragement gave me strength when I thought of giving up.

ACKNOWLEDGEMENT

God never spoils any effort. 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 family specially to my **Grand Parents** and to my **Parents** for all they have done for me over the years for the completion of this work. I am cordially grateful to my **Brother** and **Sisters** for their love, concern, untiring care, encouragement. 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 solely goes to unmatchable love and the unremitting prayers of my beloved mother and father.

I acknowledge my sincerest gratitude to honorable supervisor Associate Professor **Dr. Sabika Firasat,** for her keen observations, continuous guidance, ultimate help, encouragement, valuable time and great efforts during all stages of this work. Doubtlessly, it was all because of her consistent supervision that I could be able to complete it on time.

I am cordially grateful to **Dr. Sumera Altaf, Dr. Mehreen Tanveer, Dr. Amt-Ul-Aziz, Ambreen, Khizar, Muhammad Zubair** and all the patients at Alshifa Eye Trust Hospital, Rawalpindi, Pakistan for their ultimate help and persistent support during sample collection.

I would like to pay my gratitude to my research fellow **Tahira Khan,** to my seniors **Raeesa, Maria, Rutaba, Tufail, Aleesha** and to my juniors **Ayesha** and **Kashif** for their persistent support offered throughout this thesis and without whom this research work would not have been possible.

I am also thankful to my best friends **Nadir Akhtar, Muhammad Idrees, Sajjad Ali, Dr. Aitisam Ullah Shah, Raihan Ullah Khan, Muhammad Murtaza, Zia Ullah** and **Dr. Nouman Gul** and for their continuous help and support during all these years.

Asfandyar Ahmad Khan

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ABBREVIATIONS OF TERMS AND ACRONYMS

PDE6A	Phosphodiesterase 6 Alpha
Arg	Arginine Amino Acid
Ser	Serine Amino Acid
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–lindau 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
POD	Pale Optic Disc
AA	Attenuated Arteries
MR	Mental Retardation
HT	Hypertension
HL	Hearing loss
CB	Colour Blindness
IS	Impaired speech
PD	Polydactyly

ABSTRACT

Retinitis pigmentosa (RP) is a genetic disorder characterized by the progressive degeneration of retina of eye mainly the photoreceptor layer. Approximately more than 2.5 million peoples are affected from RP across the globe. Its estimated prevalence is about 1:4000 worldwide. Eye examination have revealed, weakening of retinal vessels, abnormal fundus with bone spicules accumulation, waxy pallor of optic disc, macular degeneration, and arterial attenuation. The age of onset of RP, varies from early childhood to late adulthood. The severity of RP also varies from mild unnoticeable loss of visual field to tunnel vision and impaired central vision. The mode of inheritance of RP can be autosomal dominant RP, autosomal recessive RP or X-linked RP. RP may exist in syndromic and non-syndromic forms. It is reported that, autosomal recessive RP is the most common form of retinal dystrophies in Pakistani population. This study was performed to analyze a common mutation of *PDE6A* gene i.e. c.304C>A, p.R102S, in twenty four consanguineous RP families recruited from different regions of Pakistan. Ethical approval was obtained from the Bio-ethical review committee, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Pakistan and Al-Shifa Trust Eye Hospital Rawalpindi, Pakistan. All the enrolled families were diagnosed with RP by ophthalmologist. Participating members were interviewed for family history of disease, pedigree drawing, and clinical records were collected. Blood samples were collected from affected and unaffected members after written consent. The genomic DNA was extracted for genetic analysis. Primers were designed to amplify hotspot exon 1 of *PDE6A* gene for mutational analysis. After the amplification of selected exon, the amplified products were purified and sent for Sanger's sequencing. Sequencing data analysis did not identified previously reported missense variant c.304C>A causing (p.Arg102Ser) in exon 1 of *PDE6A* gene in any of the enrolled families. However, other previously reported and novel variants were identified in exon 1 of *PDE6A* in enrolled RP families. For families in which no disease-causing variant was identified, screening of remaining exons of *PDE6A* and other reported genes be performed to identify molecular genetic defect. Results of this study also showed that consanguinity contributes to high incidence of recessively inherited disorders including RP in our population. For this reason, genetic counselling was provided to all participating families.

Chapter No. 1:

INTRODUCTION

1.1. Structure of Human Eye:

The human`s eye is a delicate and complex structure. It is designed to collect substantial amount of visual information about the surrounding environment (Buscemi *et al.*, 2018). The human`s eye consists of three regions which collectively perform the function of visual sensation (Hoshino *et al.*, 2017). These regions are; outer region, middle region and inner region.

The outer region of eye consists of sclera and cornea. The sclera work as a coat of connective tissue which perform the function of protection from external and internal forces and helps to main the shape of eye (Sridhar, 2018). The cornea performs the function of refraction and transmission of light to lens and retina. It also provides the protection from structural damage to the inner parts of eye and different infections (Altinkurt *et al.*, 2021). The sclera and cornea are connected at limbus. The visible portion of sclera is enclosed in a transparent mucous membrane which is called as conjunctiva (Boote *et al.*, 2020).

The middle region of eye consists of iris, ciliary body, and choroid. Iris regulates the amount of light which reaches to the retina by controlling the size of pupil (Aguirre, 2019). Ciliary body controls shape and power of lens. It is also the site to produce aqueous. Choroid is a vascular layer which supply nutrients and oxygen to the outer layers of retina (Murugan, 2019).

The inner region of eye consists of retina and complex structure of neurons. They collectively capture and process the light which enters to the eye (Menon *et al.*, 2019). There are three transparent structures in the eye. These are; lens, vitreous and aqueous, which are surrounded by ocular layer (Wilson *et al.*, 2020).

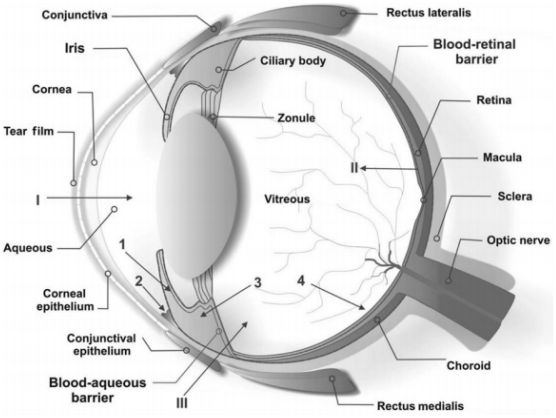


Fig.1.1: Structure of Human Eye

1.1.1. Anterior segment:

Anterior segment of human eye consists of cornea, iris, crystalline lens, ciliary muscles and aqueous humor (Lu *et al.*, 2021). It is the portion of the eye which is visible to observer without using special instruments. It is considered as the front segment of the eye (Ang *et al.*, 2018). It contains most of those structures of eye which are responsible for the image focusing on retina of eye (Park *et al.*, 2018).

The main focusing structure of the eye is cornea. It is responsible for the 75% focusing power of eye. The remaining power of focusing is provided by the crystalline lens and serve the function of further refinement (Perez-Garmendia *et al.*, 2020). The lens also allows the eye to focus on the distantly different placed objects from eye (Ang *et al.*, 2018).

The pupil or aperture of the eye for the regulation of different intensities of light is controlled by the iris. The iris is basically an extension of ciliary body (Taradaj *et al.*, 2018). Ciliary body is found in the anterior segment of eye and is responsible for multiple functions (Park *et al.*, 2018). It produces a fluid called aqueous humor which fills the anterior segment of eye and controls the shape of lens in eye (Ang *et al.*, 2018).

1.1.2. Posterior segment:

Posterior segment of human eye consists of retina and vitreous humor (Burhan *et al.*, 2021). Retina is the inner most portion of the posterior segment. It is the site where the image formation takes place (de Oliveira *et al.*, 2020). Highly specialized tissues are present in retina

which are involved in the initial processing of image (A. S. Li *et al.*, 2021). Vitreous is a gel that fills the posterior segment of the eye. It helps in light transmission and also protects retina (K. Nayak *et al.*, 2020).

1.2. Anatomy of Retina:

Retina is a transparent, thin and sensitive tissue. It is designed to capture the light in the form of photons and to initiate the initial processing of image which is finalized by the brain (Cowan *et al.*, 2020). The retina consists of 10 layers with an average thickness of 250 µm (Hoshino *et al.*, 2017). From surface of retina to the back of human eye, the arrangement of layers are; inner limiting layer, nerve fibers layer (contains axons of ganglion cells), ganglion cell layer, inner plexiform layer (serves as a synapses between amacrine cells and ganglion), inner nuclear layer (interplexiform cells and amacrine along with spanning glial cells of retina), outer plexiform layer (serves as synapsis between horizontal, bipolar and photoreceptors cells), outer nuclear layer of photo receptor cells, outer limiting membrane, receptor layer (inner and outer segments of photoreceptors) and retinal pigment epithelium layer (RPE) (Behar-Cohen *et al.*, 2020).

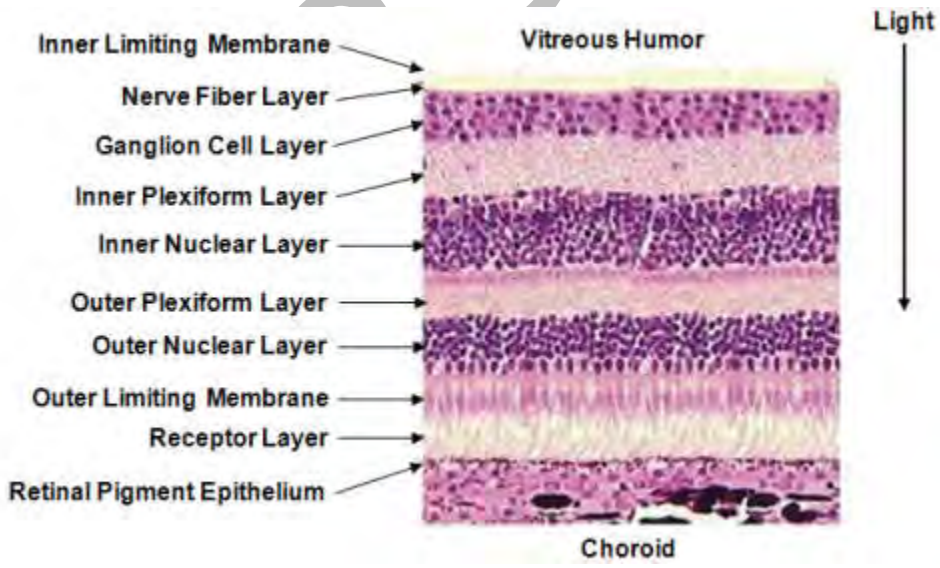


Fig.1.2: Layers of Retina of Human Eye

The retinal pigment epithelium is the first layer of retina. It serves an important role in the metabolism for the other segments of eye (Parchand *et al.*, 2021). It also act as a site for light sink where the incoming photons reduces intra-ocular glare (Caceres *et al.*, 2020).

The photons of light penetrate deeply across all the layers of retina as the receptors lies deep in retina (Stockinger *et al.*, 2020). The receptors within the retina receive the photons and convert them into the neural signals. These signals are processed through network of horizontal, amacrine, bipolar and ganglion cells (Caporarello *et al.*, 2019).

A nerve fiber is formed by the axons of ganglion cells. By which the information at optic nerve, exits from the eye (Flockerzi *et al.*, 2020). The nerve transmission from the eye is basically an intricated interconnections of many neural cells inside the retina that receive and process the initial visual information and then send them to brain for further processing (Hirawat *et al.*, 2020).

There are two types of cells in receptor layer; Rods and Cones. These are named based on their shape (X. Chen *et al.*, 2020). The outer region of receptors cells contains molecules of visual pigment called as opsins, which is sensitive to light. The rods are present in stacked disc manner while cons are present in invaginations form (Guadagni *et al.*, 2019). Rods and cones both contains the light sensitive pigments for visuals. A normal adult retina contains about 92 million rods and 5 million cones (Thoreson *et al.*, 2019). Cones are responsible for the colour recognition and fine detailed vision. They are concentrated in the central portion of retina. Rods are responsible for the low light vision and peripheral vision. They are concentrated in the mid and peripheral retina (Fain *et al.*, 2018).

At the posterior most segment of the eye a region called a macula lutea is present where the received light is focused. It is about 5-6 nm in diameter (Kritika Nayak *et al.*, 2018; Thareja *et al.*, 2022). This region has greatest density of lutein and zeaxanthine which are light sensitive pigments. These pigments protect the retinal neural cells from oxidative stress (Johra *et al.*, 2020). Within macular region the fovea centralis is present. It is a small region at the middle of retina where the vision takes place. It I about 1.5 mm in diameter and without rods, only cones and neural layers are present (Mrowicka *et al.*, 2022). The area which is located outside to fovea is called parafoveal region. It is the region where the transition of retina takes place from cone-domination to rod-domination (Escobar-Villado *et al.*, 2021; Warner *et al.*, 2020).

The two sources provide nourishment to the retina are, retinal vasculature and choroidal vasculature (located in between the sclera and retina) (Ludwig *et al.*, 2022). The retinal

vasculature provides nourishment to the inner layers of retina while the choroidal vasculature provides nourishment to the retinal outer layers and RPE (metabolically active) (Hurley, 2021).

The most common condition which affects the retina and visual sensation is the age-related macular degeneration (ARMD) (Stahl, 2020). It is characterized by the loss of vision and light sensation at the center of visual field. During the light processing process, waste is produced by the photoreceptor cells which are eliminated by the RPE (Al-Zamil *et al.*, 2017). In ARMD, ability of RPE to remove waste from photoreceptor cells is reduced. As a result of which the waste starts to accumulate in photoreceptor cells in the form of "drusen" (Heesterbeek *et al.*, 2020). They disrupt the metabolic activities of retina and as a result the retina starts to deteriorate. When the blood vessels of choriocapillaris breaks this condition is called as "wet ARMD" and this is significantly worse (Thomas *et al.*, 2021). The earlier signs of ARMD are detectable by eye examination. ARMD is generally hereditary (Cowan *et al.*, 2020; Somasundaran *et al.*, 2020).

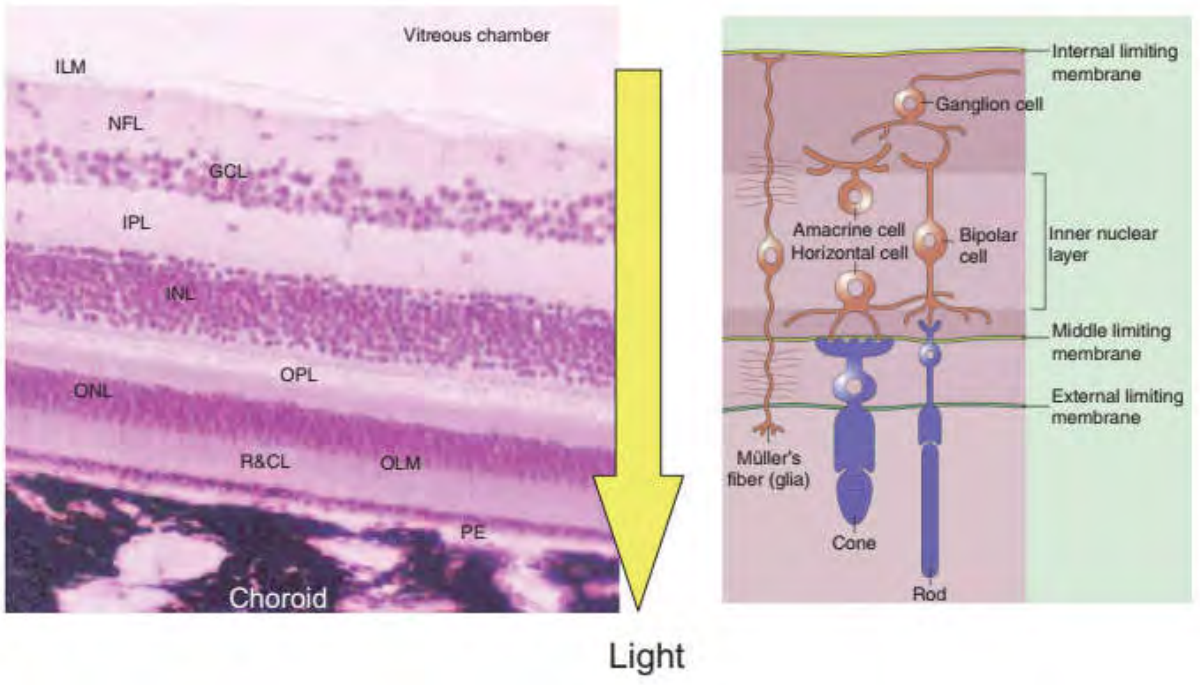


Fig.1.3: Structure and Distribution of Retinal Cells of Human Eye

1.3. Retinal disorders:

In most of the human retinopathies, such as Vision impairment, Macular degeneration (MD) and Retinitis pigmentosa (RP) are due to the dysfunction or degeneration of the photoreceptors (Bruninx *et al.*, 2020). Some of the most important retinal disorders are;

i. Retinoblastoma (RB)
ii. Diabetic retinopathy (DR)
iii. Age-related macular degeneration (AMD)
iv. Retinal detachment (RD)
v. Acute idiopathic blind-spot enlargement syndrome (AIBSES)
vi. Von hippel–lindau disease (VHLD)
vii. Tuberous sclerosis (TS)
viii. Cone dystrophy
ix. Retinitis pigmentosa (RP)

1.3.1. Retinoblastoma (RB):

Retinoblastoma (Rb) is a rare cancer of retina of the eye. It is found in about 1:15000 population worldwide (Al-Zamil *et al.*, 2017). It is an intraocular malignant tumor develops in early childhood or infancy. Worldwide it is diagnosed in about 8,000 infants each year (Heesterbeek *et al.*, 2020). It occurs when both the alleles of retinoblastoma gene (RB1) are mutated. When primitive retinal cells transform into malignant then it cause tumor formation. The tumor is mostly appears before the age of 3 years (Thomas *et al.*, 2021). The children from a family having positive history of RB should examine the retina till 3 years on monthly basis.

There are two forms of retinoblastoma: non-heritable and heritable. Heritable form comprises of about 40% of total Rb cases and having autosomal dominant pattern with variable degree of phenotypic expression (Heesterbeek *et al.*, 2020). It usually occurs bilaterally and multifocal. Affected children may have risk of osteosarcoma, brain tumor, lungs cancer and melanoma. Non-heritable form usually occur in unilateral form and the affected persons are not at high risk of other malignancies (Al-Zamil *et al.*, 2017; Stahl, 2020). The most important sign that confirms the diagnosis of Rb is the presence of leukocoria (white pupil), which can be seen on eye`s photographs (Rishi *et al.*, 2020).

1.3.2. Diabetic Retinopathy (DR):

Diabetic retinopathy (DR) is a condition in which the patient having either type 1 (insulin dependent) or type 2 (non-insulin dependent) diabetes mellitus develops retinal complications (Lechner *et al.*, 2017). These complications include the reduction in capillary flow, deformation of RBCs, degeneration of pericytes and proliferation of endothelial cells (W. Wang *et al.*, 2018). These factors results in the increase hypoxia in the retina and leads to the production of vascular endothelial growth factor (VEDF). High level of VEGF causes the breakage of blood-retina barrier and lead to the diabetic macular edema (DME) as well as diabetic retinopathy (Kang *et al.*, 2020; Lechner *et al.*, 2017). It is divided into two categories: Proliferative diabetic retinopathy (PDR) and Non-proliferative diabetic retinopathy (NPDR) (Azad *et al.*, 2021).

Worldwide, 80% of diabetic retinopathy`s cases are more than the age of 10 years. It is one of the leading causes of the blindness worldwide. The most important risk factor of this is the duration of disease (Simó-Servat *et al.*, 2019). In case of type 1 diabetes the risk of diabetic retinopathy is about 50% after the age of 10 years. This risk increases to 90% after the age of 30 years. In case of type 2 diabetes its risk is about 5%, which increases to 8% after 10 years with loss of vision in some cases (W. Wang *et al.*, 2018; Youngblood *et al.*, 2019). Other risk factors includes; arterial hypertension and poor metabolic control. Strict blood sugar regulation is effective in the development and progression of diabetic retinopathy (Vujosevic *et al.*, 2020). DR is mostly asymptomatic at early stages of onsets and regular eye examination is required for diabetic patients for timely diagnosis (Fung *et al.*, 2022).

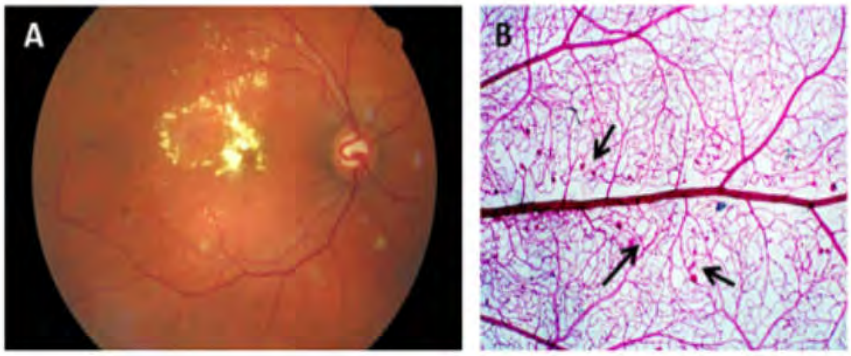


Fig.1.4: Fundus Photograph of Patient having Diabetic Retinopathy

1.3.3. Age-Related Macular Degeneration (AMD):

Age-related macular degeneration (AMD) is a condition in elder age involving retina, retinal pigment epithelium layer (RPE) and choroid (Tan *et al.*, 2020). Mostly the center of retina also known as macula is affected with leads to vision impairment (Stahl, 2020). Usually, it is a bilateral (affect both eyes) condition with variable severity. This arise by the increase in the deposition of waste material (lipofuscin containing compounds) in RPE beneath retina (Kaarniranta *et al.*, 2020; Nashine, 2021).

There are two forms of AMD: Non-neovascular AMD and Neo-vascular AMD. The non-neovascular form (dry AMD) is the earlier stage of AMD which can be progress to exudative or neovascular form (wet AMD) (Jaffe *et al.*, 2021). Deposits of yellowish residues can be seen on retina and named as "drusen". These residues can leads to the progressively developed atrophy of RPE and retina (Al-Zamil *et al.*, 2017).

In case of non-neovascular form the signs and symptoms includes; progressively decrease in visual acuity, reading difficulty, increase light sensitivity and enlargement in the atrophy area (García-Layana *et al.*, 2017; Stahl, 2020). While in case of neovascular form the signs and symptoms includes; pathologically arising of new vessels from the choroid region, decrease in visual acuity and leakage of flid in the region of retina and RPE which leads to distortion. If the patient is left untreated then it may leads to the further reduction in vision (Al-Zamil *et al.*, 2017; Ashraf *et al.*, 2018).

Risk factors for AMD contains; age, smoking, increase body mass index (BMI), and excess fat intake (Heesterbeek *et al.*, 2020). Its prevalence has been increased in last 2-3 decades and will continue to increase in future. The reason for this hike are many but the most important is the increase in the aging population worldwide. The prevalence of AMD was 1.75 million in 2003, 3.13 million in 2010 and 7.65 million in 2020 worldwide (Armento *et al.*, 2021; Flores *et al.*, 2021).

1.3.4. Retinal Detachment (RD):

Retinal detachment (RD) is a condition in which the vitreous fluid enters through hole or retinal tears and leads to the detachment of retina from choroid (Kwok *et al.*, 2020). Lifetime risk of this condition is approximately 0.1% but higher in those patients who are old, myopic, having positive family history of RD or having ocular trauma (Nagpal *et al.*, 2018).

Patients having RD frequently have loss in visual field and a dark shadow appear in peripheral vision. It can be diagnosed by the dilated eye examination which clarify whether the macula (central retina) is involved or not involved (Iaccheri *et al.*, 2021; Qureshi *et al.*, 2020). If macula is involved, then the visual acuity is lost in central region while if the macula is not involved then the person needs urgent medication in order to prevent further progression of RD which may effect the central vision (Grabowska *et al.*, 2021).

RD is difficult to treat due to its uniqueness and variation in location, duration, size and age of the patient. Reattachment of retina by surgical procedures is the only way to Improve the visual field and vision (Kunikata *et al.*, 2019).

1.3.5. Acute Idiopathic Blind-Spot Enlargement Syndrome (AIBSES):

Acute idiopathic blind-spot enlargement syndrome (AIBSES) is a rare disorder of outer retinopathy. It was first reported by Eletcher in 1988 (Gunasagaran *et al.*, 2022). This condition is characterized by the blind spot enlargement that may be isolated or associated with chorioretinopathies (acute macular neuroretinopathy, acute zonal occult retinopathy, multifocal inner choroiditis and multiple evanescent white dot syndrome) (Quinones *et al.*, 2020; M. Wang *et al.*, 2018). Recent studies shows that this condition is a spectrum of PICCP (Primary Inflammatory Choriocapillaropathy). It has overlapping clinical features with PICCP. It is commonly found in adult myopic women (Gunasagaran *et al.*, 2022; M. Wang *et al.*, 2018). It is diagnosed with the visual field loss and abnormal values on electroretinogram (ERG) (Quinones *et al.*, 2020).

1.3.6. Von Hipple-Lindau Disease (VHLD):

Von hippel–lindau disease is a genetic disease which is due to a defect located on the short arm of chromosome number 3 (Varshney *et al.*, 2017). It is characterized by the production of malfunctioned proteins that leads to the abnormal development of cysts, tumors and new blood vessels in various parts of the eye specially retina (Gläsker *et al.*, 2020; Wiley *et al.*, 2019). It is an autosomal dominant disease. According to two-hit theory, it require alteration of two copies of the gene for abnormal growth (Gläsker *et al.*, 2000; Jonasch *et al.*, 2021). Such kind of mutations and abnormal growth are occur in the tissues of kidney, brain and retina. It can occur at any stage of patient`s life (Karimi *et al.*, 2020).

1.3.7. Tuberous Sclerosis Disorder (TSD):

Tuberous sclerosis is an autosomal dominant disorder which is caused by the mutation in any one of two TSC (Tuber sclerosis complex) genes i.e. TSC1 and TSC2 gene (Uysal *et al.*, 2020; Zamora *et al.*, 2022). TSC1 is located on long arm of chromosome number 16 and produce hamartin protein while TSC2 is located on short arm of chromosome number 16 and produce tuberin (Javaid *et al.*, 2019). It is characterized by the presence of calcified astrocytic retinal hamartoma near or at optic disc (Portocarrero *et al.*, 2018). The normal astrocytes are responsible for the support and protection of retinal neurons and also play its role in maintain the normal functions of retina (Northrup *et al.*, 2021). The calcified astrocytic retinal hamartoma is multilobulated surface and resembles the mulberry. This appearance is the key feature for the diagnosis (Dzefi-Tettey *et al.*, 2021). Flat translucent smooth tumors are also found in retina which also represents the astrocytic hamartomas in the TSD patients (Mitchell *et al.*, 2021).

1.3.8. Cone dystrophy:

Cone dystrophy is a heterogenous group of inherited disorders that leads to cone dysfunction and the post reception pathway (Birtel *et al.*, 2018). The common clinical signs and symptoms that are associated with cone dystrophy includes, photophobia, nystagmus, colour vision and visual acuity impairment (L. Jaffal *et al.*, 2022). It may be progressive or stationary.

The progressive cone dystrophy is usually developed at childhood or early in adulthood while the stationary cone dystrophy are congenital and the dysfunctional cones are present at birth (Gill *et al.*, 2019). On the basis of mode of inheritance they can be categorized as; autosomal dominant, autosomal recessive and X-linked cone dystrophy (De Silva *et al.*, 2021; Hadalin *et al.*, 2021). At initial stages the fundus photographs shows macular degeneration which may leads to pigmentation and peripheral atrophy (Birtel *et al.*, 2018). The progressive cone dystrophy may lads to progressive rod dystrophy. Therefore, in some cases both are overlapping and can be associated with RP (Nassisi *et al.*, 2021).

1.3.9. Retinitis pigmentosa (RP):

The term Retinitis pigmentosa (RP) was used by F.C Donder (Dutch ophthalmologist) in 1857 (Bruninx *et al.*, 2020). His colleague A.C. van Trigt described the RP three years earlier in the form of retinal degeneration (Athanasiou *et al.*, 2018). It is a genetic disorder characterized by the progressive degeneration of retina of both eyes mainly the photoreceptor layer (Y. P. Li *et al.*, 2021). It is also a heterogenous group of retinal diseases which involves inherited neurodegenerative diseases that effects both structures and functions of RPE and photoreceptors (Newton *et al.*, 2020). The first case of RP was reported in 1853 by F.A. von Ammon and M. Schon in Netherland (Ducloyer *et al.*, 2020). Approximately more than 2.5 million peoples are affected from RP worldwide. Its prevalence is about 1:4000 worldwide (Coussa *et al.*, 2019; Daggula *et al.*, 2020). The term “retinitis”, although is inaccurate because there is no inflammation involved but this term is used globally. In this condition there is a gradual apoptotic cell loss which leads to the reduction in the retinal functions and ultimately cause retinal atrophy and blindness (S. K. Verbakel *et al.*, 2018; M. Xu *et al.*, 2020).

1.3.9.1. Pathophysiology of retinitis pigmentosa:

There are two kind of photoreceptor cells which are mainly responsible for the vision in low and high intensities of light (Colombo *et al.*, 2021). These are rod cells and cone cells. The rod cells are responsible for the dim light and peripheral vision while the cone cells are responsible for the bright light and central vision (de Bruijn *et al.*, 2020; Lang *et al.*, 2019). In case of RP, the rod cells are affected initially and then it leads to the degeneration of cone cells.

Thus, the more accurate term used for RP is Rod-Cone dystrophy (Strong *et al.*, 2017; Wheway *et al.*, 2020).

Visual impairment appears in initial stages with certain degree of night blindness (NB) and loss of visual field (Sudharsan *et al.*, 2019). The severity of visual impairment and other disorders depends upon the degree of disfunction and degeneration of photoreceptor cells in retina (Meng *et al.*, 2020). The loss of peripheral visual field will commonly remain unnoticed by patients until the central part get affected. The central vision will loss in the late stages of disease (Lunghi *et al.*, 2019). The age of onset of RP, varies and depends upon its type. Mostly it ranges from the early childhood to the late adulthood (Kim *et al.*, 2021; Murakami *et al.*, 2020). The severity of RP also varies from mild unnoticeable loss of visual field to tunnel vision and impaired central vision. The most common pattern of RP is that it is steady and slowly deterioration of retina or RPE (Gagliardi *et al.*, 2019).

1.3.9.2. Types of RP:

There are two major types of Retinitis pigmentosa (RP) i.e. Syndromic RP and Non-Syndromic RP. Based on mode of transmission, RP can be categorized as Autosomal dominant RP (adRP), Autosomal recessive RP (arRP) and X-Linked RP. Autosomal recessive RP (arRP) is the most frequently reported form of RP (Cehajic-Kapetanovic *et al.*, 2020; Kuehlewein *et al.*, 2021). It is frequently reported in Saudi, South Indian, Israeli and Pakistani population with high rate of endogamy and consanguinity (Dan *et al.*, 2020).

A. Syndromic RP:

In Syndromic RP, there is an association of other syndromes with the onset of RP. There are more than 50 syndromes which are associated with the RP (McConnachie *et al.*, 2021). It involves multiple organs and has pleiotropic effect in which most of the time other syndromes are affecting primarily while the retinal disorder is secondary in pathology. RP is mostly non-syndromic (Shamseldin *et al.*, 2020). Among all the cases of RP, approximately 25-35% will be syndromic (Fuster-García *et al.*, 2021). Most important syndromes which are associated with RP are; Usher syndrome and Bardet-Biedl syndrome. Other associated syndromes includes; Refsum

syndrome, Alstrom syndrome, Wolfram syndrome, Cockayne syndrome, Flynn Aird`s syndrome, Kearn-Sayre syndrome (Colombo *et al.*, 2021; O'Neal *et al.*, 2022; Zeviani *et al.*, 2021).

Usher`s syndrome is the most frequent syndrome associated with RP. It is characterized by the congenital hearing impairment followed by RP development in early teenage (Toms *et al.*, 2020). Bardet-Biedl syndrome is the second most frequent syndrome associated with RP. It is characterized by polydactyly, renal abnormalities, mental retardation and obesity (Huang *et al.*, 2021). Other syndromic conditions which are mentioned above, includes RP as its component (Nuzbrokh *et al.*, 2021).

B. Non-Syndromic RP:

In Non-syndromic RP, there is no associated syndrome. The disease can be diagnosed by common signs and symptoms without any complication (Nwosu *et al.*, 2020). About 65-75% of reported cases are of non-syndromic RP where only eye can be affected. U.S have been reported about 6500 non-syndromic RP cases in last 3 years (S. K. Verbakel *et al.*, 2018). Among all the reported cases, 30% cases are of adRP, 20% are of arRP, 15% are of X-linked RP, 5% are of recessive LCA and the reaming 30% are of isolated RP (O'Neal *et al.*, 2022; Zeviani *et al.*, 2021). The isolated cases have mostly autosomal recessive mutations but de novo mutations such as dominant cases are also reported in patients.

1.3.9.3. Prevalence:

Globally, 285 million people are suffering from vision impairment. Among them about 2-3% are affected with Retinitis pigmentosa (Colombo *et al.*, 2021). RP has a worldwide prevalence. Approximately more than 2.5 million peoples are affected from RP worldwide. Its prevalence is ranging from 1:4000 to 1:9000 depending upon the demography of area (Gao *et al.*, 2021; Toms *et al.*, 2020). Generally, its prevalence is about 1:4000 worldwide. Among all the cases of RP, approximately 25-35% will be syndromic while 65-75% of reported cases are of non-syndromic RP where only eye can be affected (Menghini *et al.*, 2020; Teo *et al.*, 2021). In 65-75% of reported non-syndromic cases, 30% cases are of adRP, 20% are of arRP, 15% are of X-linked RP, 5% are of recessive Leber congenital amaurosis (LCA) and the reaming 30% are of isolated RP (Gao *et al.*, 2021; Piotter *et al.*, 2021; Schwartz *et al.*, 2020).

Table 1.1: Prevalence of Different forms of Retinitis Pigmentosa

Category	Type	Percentage of Total
Syndromic and Systemic RP	Usher Syndrome	10%
	Bardet-Biedl Syndrome	5%
	Other Syndromes	10%
	Unknown types of RP	10%
Sub Total (A)		**35%**
Non-Syndromic RP	Autosomal Dominant RP	20%
	Autosomal Recessive RP	13%
	X-Linked RP	8%
	Isolated or Unknown RP	20%
	Leber Congenital Amaurosis	4%
Sub Total (B)		**65%**
Grand Total (A+B)		**100%**

In past decades, a rapid increase in RP cases has been reported. U.S have been reported about 6500 non-syndromic RP cases in last 3 years (Cross *et al.*, 2022). In 2010, the number of RP cases reported in South Asia are, 15,482 cases in Afghanistan, 58,963 cases in Bangladesh, 1603 cases in Bhutan, 391,570 cases in India, 9318 cases in Sri Lanka and 58,528 cases in Pakistan (Bouzidi *et al.*, 2021; Sun *et al.*, 2021; Thapa *et al.*, 2020). In 2020, an increase in these numbers have been reported such that, 21,821 cases in Afghanistan, 83,293 cases in Bangladesh, 3887 cases in Bhutan, 588,320 cases in India, 13,931 cases in Sri Lanka and 92,762 cases in Pakistan (M. Karali *et al.*, 2019; Van Cauwenbergh *et al.*, 2017).

It is reported that, autosomal recessive RP is the most common form of retinal dystrophies in Pakistani population (Moore *et al.*, 2020). Studies on Pakistani population shows that 64% cases are of autosomal recessive retinitis pigmentosa, 18% are of autosomal recessive Leber Congenital Amerosis, 8% are of autosomal recessive congenital stationary night blindness and 10% are of autosomal recessive cone-rod dystrophy (Maria *et al.*, 2015; Shahzad *et al.*, 2013). Totally, 150 mutations and more than 60 genes are reported to be associated with RP worldwide. Most frequently reported genes associated with RP in Pakistani population are *CRB1*,

PDE6A, *PDE6B*, *AIPL1*, *TULIP1*, *RP1*, *RPGRIP1*, *LCA5* and *SEMA4A* (Kannabiran *et al.*, 2022; Zafar *et al.*, 2017).

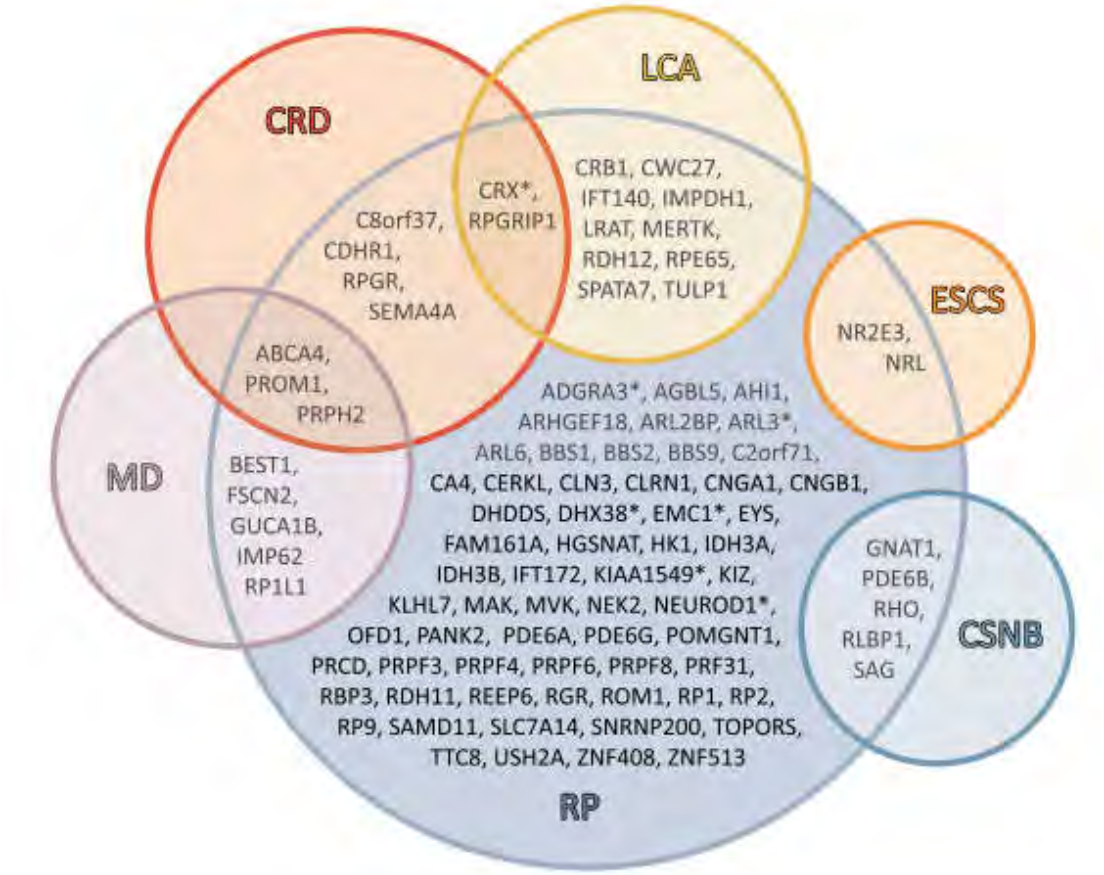


Fig. 1.5: Venn diagram showing genetic overlap between RP and other inherited retinal dystrophies (Verbakel *et al.*, 2018).

1.3.9.4. Clinical Findings, Signs and Symptoms:

Visual impairment appears in initial stages with certain degree of night blindness (NB) and loss of visual field (Asano *et al.*, 2021). The severity of visual impairment and other disorders depends upon the degree of disfunction and degeneration of photoreceptor cells in retina (Chatterjee *et al.*, 2021; Colombo *et al.*, 2021). The loss of peripheral visual field will commonly remain unnoticed by patients until the central part get affected. The central vision will loss in the late stages of disease (Downes *et al.*, 2020; Hartong *et al.*, 2006).

Night blindness or Nyctalopia is the first sign of retinitis pigmentosa. Which indicates the damage and progressive death of rod photoreceptor cells (Huang *et al.*, 2018). It is followed by the loss of peripheral vision indicating the loss of rod cells from peripheral region of retina and leads to loss of central vision in later stages (Kim *et al.*, 2021). Impairment of chromatic discrimination is also a sign of RP which indicates that the degeneration has progressed to the con cells. Ultimately, all these conditions lead to blindness (Kuehlewein *et al.*, 2021; Mizobuchi *et al.*, 2019).

Eye examination have revealed, weakening of retinal vessels, abnormal fundus with bone spicules accumulation, macular degeneration, and reduction in visual field as diagnostic sign and symptoms of RP (Rocco *et al.*, 2018; Sun *et al.*, 2020). The severity of pigmentation, arterial attenuation and macular degeneration is variable among patients (Nakazawa *et al.*, 2019).

1.3.9.5. Age of onset and rate of 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). Mostly it ranges from the early childhood to the late adulthood. The age of onset of RP is not an accurate way to find the severity of disease because it gives fewer details about the actual time at which the photoreceptors start to degenerate (Wheway *et al.*, 2020). In some cases, no symptoms were reported until mid-adulthood while in other symptomatic loss of vision appear at earlier ages of childhood (Yusuf *et al.*, 2019). If the symptoms appear earlier, then it is an indication of rapid progression of RP. Moreover, inheritance pattern also plays an important role in severity of RP (L. Kuehlewein *et al.*, 2020). Generally, X Linked form of RP is more severe as compared to autosomal recessive RP (Dan *et al.*, 2020).

1.3.9.6. Diagnosis:

RP can be diagnosed based on clinical symptoms, retinal findings, 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 boney spicule pigmentation in peripheral region of retina (A. A. Khan *et al.*, 2021). The defect in the visual field starts from the peripheral region at initial stages and progress towards the central region in final stages of RP (Chatterjee *et al.*, 2021). The most efficient diagnostic tool for RP is ERG. It

gives a clear picture of the extent and population of normal and affected retinal cells. It measures the response of photoreceptor cells in the form of electrical signal and depends on intensity of light stimulus (de Bruijn *et al.*, 2020). In dim light conditions, mainly the rods are contributing to the electrical signals while in bright light, mainly cones are contributing to the electrical signals. This variation in the conditions provides the basis for ERG diagnosis (Arsiwalla *et al.*, 2020).

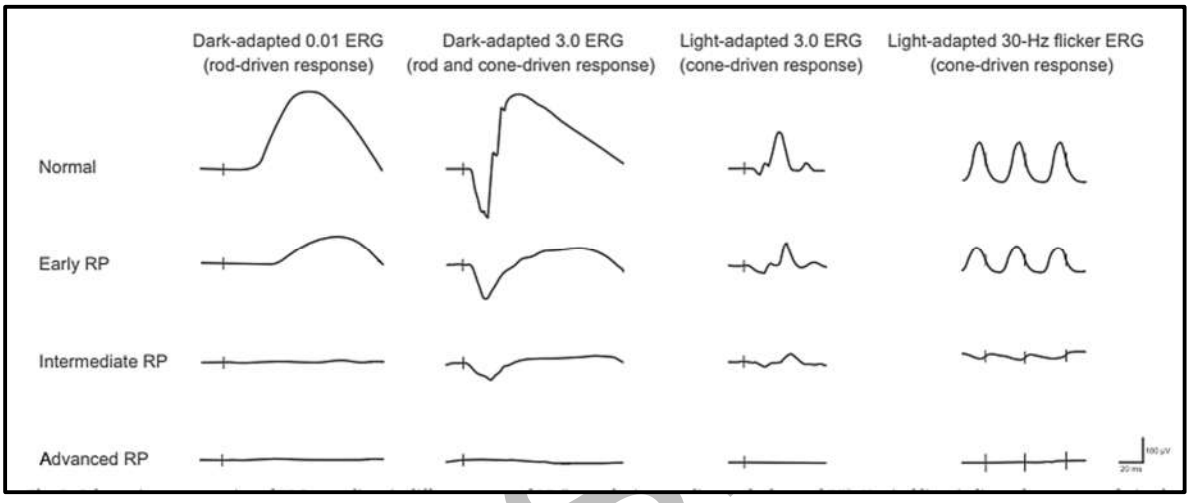


Fig. 1.6: ERG representation at different stages of Retinitis pigmentosa (Verbakel *et al.*, 2018)

1.3.9.7. Treatment:

Retinitis pigmentosa is a genetic disorder due to this reason the drugs can only slow down the RP progression or repair the degenerated cells to some extent (Daiger *et al.*, 2013). The progressive nature of RP is of great interest. It helps in the development of therapeutic agents as the photoreceptor cells degenerate slowly (Diakatou *et al.*, 2019). Presently, all the therapeutic approaches are in the efforts to slow down the retinal degeneration process and helps the patients to cope with psychological and social impacts of blindness (Ducloyer *et al.*, 2020). Nonpharmacological medications are based on the light protection strategies indicating that several genetic pigmentary retinopathies depend upon light (Goureau *et al.*, 2020). Hyperbaric oxygen therapy has also been played significant role in survival of photoreceptor cells (Newton *et al.*, 2020).

i. Pharmacological Medications:

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

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

ii. Retinal Implant:

An implant "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). The received images are then transferred to the retina in the form of electrical signals. It helps the patients of end stage RP. It improves the ability to locate light, identify the people and to read some letters at about 9 inches distance (Cornford *et al.*, 2017).

iii. Glasses:

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

iv. Lifestyle and Dietary Modifications:

Life tyle 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 specially 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 and inherit in offspring from their parents. Mostly it is in the form of autosomal recessive pattern (Valle *et al.*, 2021). If both the parents are affected or carrier, then the offspring will be affected. If one parent is affected and other is normal then the offspring will be carrier and can pass this disease to next generation if married to a carrier or affected one (Vickers *et al.*, 2018). RP is passing from one generation to next, more frequently in families having cousin marriages. Therefore, if a family has positive history for RP, should be counselled and advised not to do cousin marriage (Wheway *et al.*, 2020). If a couple has both partners affected, then they must be advised to go for family planning or no more kids (P. Xu *et al.*, 2017).

1.4. *PDE6A* Gene and its association with RP:

Phosphodiesterase 6 Alpha (*PDE6A* or PDE6α) gene is located on "q: arm (long arm) of chromosome number 5 at position 32 i.e. Chr.5q32 (Bujakowska *et al.*, 2020). It has 22 exons and 21 introns. It is approximately 45-50 kb in size (Dawood *et al.*, 2021). The 22 exons, codes for a log protein of 860 amino acids (Li *et al.*, 2022). Globally, 40 pathogenic mutations have been reported in PDE6α gene. Among them, 26 mutations are single base substitution which constitute about 65% of all pathogenic mutations of PDE6α (Nair *et al.*, 2017; Occelli *et al.*, 2017). Remaining 14 variants (35% mutations) are non-sense and mis-sense variants. Mutations in phosphodiesterase 6α (PDE6α) gene cause malfunctioning of cGMP (Cyclic Guanosine Monophosphate) and leads to retinal degeneration ultimately associated with autosomal retinitis pigmentosa in humans (A. A. Khan *et al.*, 2021).

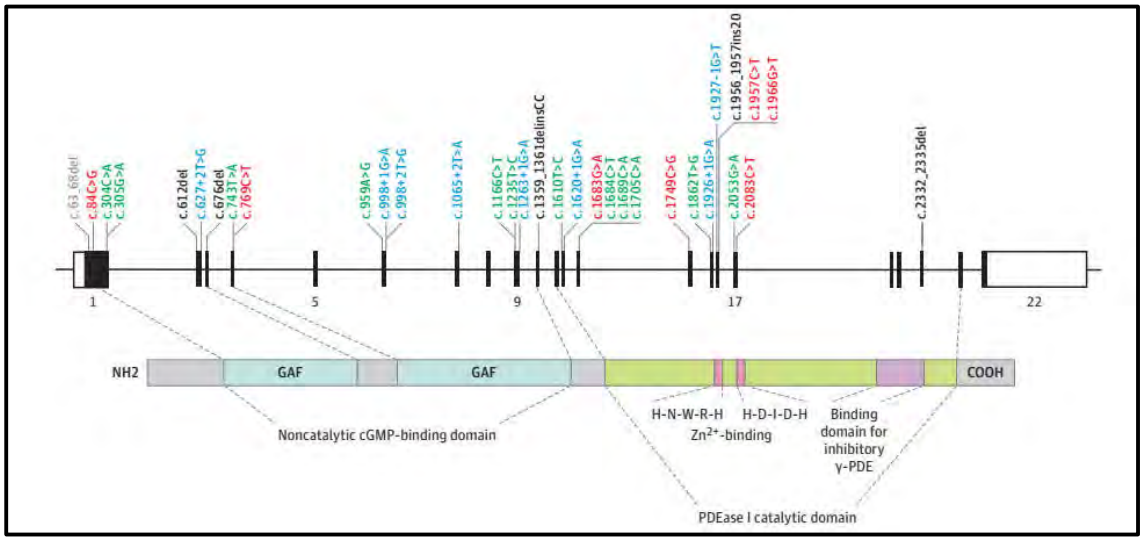


Fig. 1.7: Genomic and Protein Structure of *PDE6A* and Location of Variants (Kuehlewein *et al.*, 2020).

The phosphodiesterase 6 (PDE6) enzyme is a hetero-tetrameric protein consists of alpha, beta and two gamma subunits (Takahashi *et al.*, 2018). The alpha subunit is encoded by PDE6α or *PDE6A* gene, beta subunit is encoded by PDE6β or PDE6B gene and the two gamma subunits are encoded by PDE6γ gene or PDE6G (Schön *et al.*, 2017). The alpha subunit has mass of 88kDa, beta subunit has 84kDa and gamma subunit has 11kDa (Crouzier *et al.*, 2021). These three kinds of subunits are the essential part of cyclic guanosine monophosphate (cGMP). The cGMP play role as a second messenger molecule in the process of phototransduction and perform the function of signal transduction from retina to brain through optic nerve. It is abundantly found in retina of eye where it performs the function of phototransduction in different conditions of light by cGMP hydrolysis in the process of photoreception (Hayashi *et al.*, 2021; A. A. Khan *et al.*, 2021; L. Kuehlewein *et al.*, 2022). The alpha and beta subunits have stimulatory effect while gamma subunit has inhibitory effect on phototransduction cascade (Petersen-Jones *et al.*, 2019).

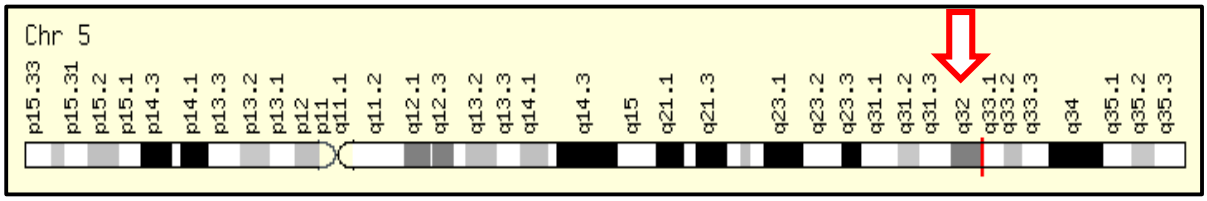


Fig. 1.8: Location of *PDE6A* Gene on Chromosome 5q32

Studies shows that, the c.304C>A, p.Arg102Ser mutation, results in the substitution of arginine amino acid with serine amino acid at 102nd amino acid position in PDE6α subunit protein (Kuehlewein *et al.*, 2021). Arginine is a polar and positively charged amino acid. When it is substituted with serine (polar but no charge) then the normal structure and functions of PDE6α protein alters (Mowat *et al.*, 2017). The mutant residue acquire small size and negative charge, thus leads to loss of interaction between GAF domain and cGMP-binding domain. As a result of which cGMP did not perform its normal function and starts to accumulate in photoreceptor cells (Dawood *et al.*, 2021). High level of malfunctioned cGMP cause stress in photoreceptor cells and leads to degeneration. This condition ultimately leads to autosomal recessive retinitis pigmentosa (arRP) in human (Dawood *et al.*, 2021; Khan *et al.*, 2021). The c.304C>A, p.Arg102Ser mutation, is associated with both types of RP i.e. Syndromic and Non-Syndromic RP (Li *et al.*, 2022).

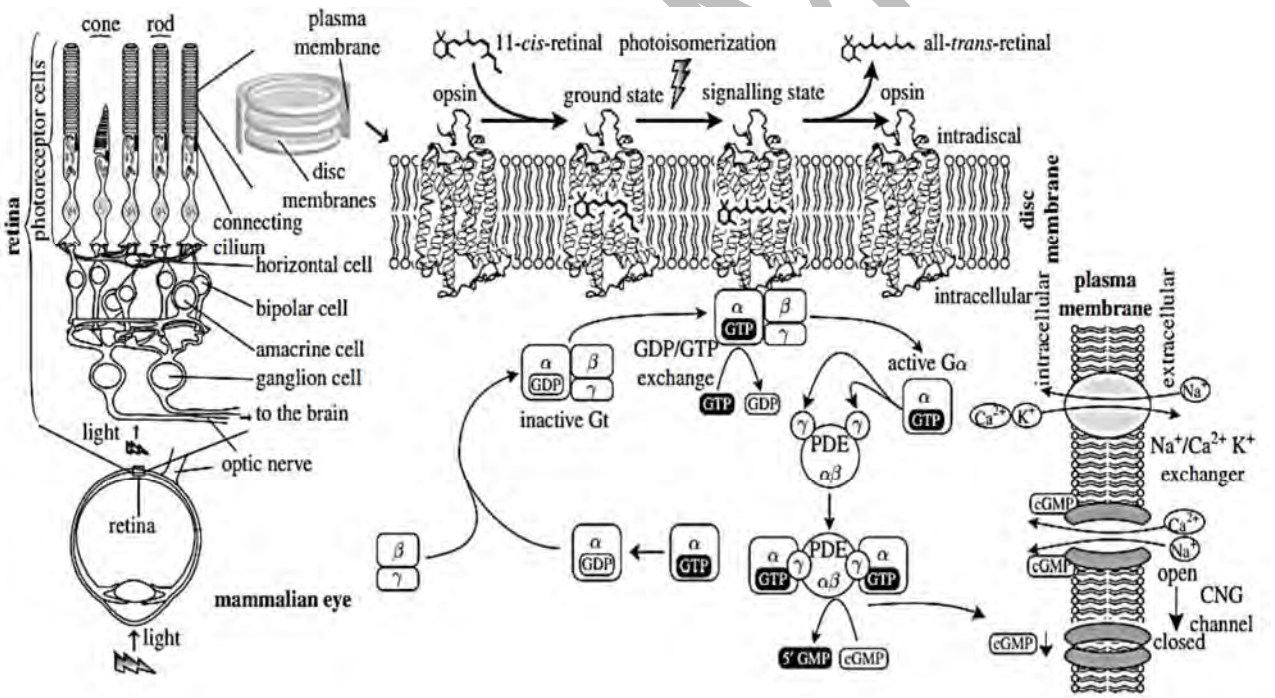


Fig. 1.9. Mechanism of phototransduction from the perception of light to photoreceptors hyperpolarization.

Table 1.2: Mutations 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.(Leu140Val)	arRP	1	4	(Haque, 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)
CLRN1	c.92C>T	p.(Pro31Leu)	arRP	1	6	(Ali *et al.*, 2008)
CNGA1	c.626_627del	p.(Ile209Serfs*26)	arRP	1	7	(Zhang *et al.*, 2004)
CNGA1	c.1298G>A	p.(Gly433Asp)	arRP	1	3	(Khan *et al.*, 2014)
CNGB1	c.2284C>T	p.(Arg762Cys)	arRP	1	5	(Haque, 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, 2022)
DHX38	c.995G>A	p.(Gly332Asp)	arRP	1	4	(Wang, 2019)
IMPG2	c.1680T>A	p.(Tyr560*)	arRP	1	2	(Yusuf *et al.*, 2019)
PDE6A	c.304C>A	p.(Arg102Ser)	arRP	2	5	(Khan *et al.*, 2021)
PDE6A	c.889C>T	p.(Gly297Ser)	arRP	1	4	(Qureshi *et al.*, 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, 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 *et al.*, 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, 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)

Table 1.3: Mutations associated with *PDE6A* gene, identified in different Ethnicities of world.

Nucleotide Change	Protein change	Exon	No. of Cases	Ethnicity	Clinical Parameters				Reference
					Syndromic	Non-Syndromic	Age of Onset	Progression	
c.1363A>T	p.(Lys455Ter)	10	04	American	Yes	No	First Decade	Progressive	(Chen *et al.*, 2018)
c.305G>A	p.(Arg102His)	1	08	American	Yes	No	First Decade	Progressive	(Riera *et al.*, 2019)
c.205C>T	p.(Gln69*)	1	02	American	No	Yes	First Decade	Progressive	(Kuehlewein *et al.*, 2022)
c.878C>T	p.(Pro293Leu)	5	07	American	No	Yes	First Decade	Progressive	(Takahashi *et al.*, 2018)
c.2333A>T	p.(Asp778Val)	20	02	American	Yes	No	First Decade	Progressive	(Daiger *et al.*, 2007)
c.1171G>A	p.(Val391Met)	9	06	American	Yes	No	First Decade	Progressive	(Li *et al.*, 2021)
c.1705C>A	p.(Gln569Lys)	13	05	American	No	Yes	First Decade	Progressive	(Khateb *et al.*, 2019)
c.1717T>C	p.(Ser573Pro)	13	03	American	Yes	No	First Decade	Progressive	(Zhang *et al.*, 2018)
c.959A>G	p.(Asn320Gly)	6	03	American	Yes	Yes	First Decade	Progressive	(Zobor *et al.*, 2022)
c.1032C>A	p.Ser344Arg	7	11	American	Yes	No	First Decade	Progressive	(Riera *et al.*, 2019)
c.1166C>T	p.(Pro389Leu)	9	08	American	Yes	Yes	First Decade	Progressive	(Li *et al.*, 2021)
c.1336delA	p.(R446Gfs8*)	10	06	American	Yes	No	First Decade	Progressive	(Kuehlewein *et al.*, 2022)
c.1681G>A	p.(Trp561Ter)	13	05	American	Yes	Yes	First Decade	Progressive	(Li *et al.*, 2021)
c.433G>A	P.(Ala145Thr)	4	02	Pakistani	Yes	Yes	First Decade	Progressive	(Riera *et al.*, 2019)
c.1749C>G	p.(Tyr583Ter)	14	04	American	Yes	Yes	First Decade	Progressive	(Wang, 2019)
c.1963C>T	p.(His655Tyr	16	09	European	No	Yes	First Decade	Progressive	(Kuehlewein *et al.*, 2020)
c.933+4C>T	splice effect	5	08	European	Yes	No	First Decade	Progressive	(Daiger *et al.*, 2007)
c.2053G>A	p.(Val685Met	17	13	European	No	Yes	First Decade	Progressive	(Wang, 2019)

c.676delC	p.(His226ThrfsX2	3	04	European	No	Yes	First Decade	Progressive	(Daiger *et al.*, 2007)
c.304C>T	p.(Arg102Cys)	1	15	European	Yes	No	First Decade	Progressive	(Kuehlewein *et al.*, 2020)
c.908C>G	p.(Ser303Cys)	5	09	European	Yes	No	First Decade	Progressive	(Li *et al.*, 2021)
c.298C>T	p.(Arg100Trp)	1	14	European	Yes	Yes	First Decade	Progressive	(Riera *et al.*, 2019)
c.784G>A	p.(Ala262Thr)	4	02	European	No	Yes	First Decade	Progressive	(Kuehlewein *et al.*, 2020)
c.923C>T	p.(Pro308Leu)	5	10	European	Yes	No	First Decade	Progressive	(Chen *et al.*, 2018)
c.769C>T	p.(Arg257*)	4	11	European	Yes	No	First Decade	Progressive	(Li *et al.*, 2021)
c.937del	p.(Ile313fs)	5	10	European	Yes	No	First Decade	Progressive	(Wang, 2019)
c.1960C>T	p.(Gln654Term	16	07	European	Yes	No	First Decade	Progressive	(Wang, 2019)
c.1268delT	p.(Leu423*)	9	03	Asian	Yes	Yes	First Decade	Progressive	(Mizobuchi *et al.*, 2019)
c.1349T > C	p. (Phe450Ser)	10	12	Asian	Yes	Yes	First Decade	Progressive	(Zhang *et al.*, 2018)
c.1246G > A	p. (Asp416Asn)	9	06	Asian	Yes	Yes	First Decade	Progressive	(Khateb *et al.*, 2019)
c.1675C>A	p.(Tyr558*)	13	05	Asian	Yes	Yes	First Decade	Progressive	(Takahashi *et al.*, 2018)
c.1684C>T	p.(Arg562Trp)	13	08	Asian	No	Yes	First Decade	Progressive	(Riera *et al.*, 2019)
c.1685G>A	p. (Arg562Gln)	13	11	Asian	No	Yes	First Decade	Progressive	(Mizobuchi *et al.*, 2019)
c.1747T > A	p. (Tyr583Asn)	14	08	Asian	Yes	Yes	First Decade	Progressive	(Takahashi *et al.*, 2018)
c.285C > A	p.(Ser95Arg)	1	04	Asian	Yes	No	First Decade	Progressive	(Mizobuchi *et al.*, 2019)
c.1630C>T	P.(Arg544Trp)	12	03	Pakistani	Yes	Yes	First Decade	Progressive	(Khan *et al.*, 2021)
c.304C>A	P.(Arg102Ser)	1	03	Pakistani	Yes	Yes	First Decade	Progressive	(Khan *et al.*, 2021)
c.1408-2A>G	p.(Lys470_Leu491 del)	10	18	Pakistani	Yes	Yes	First Decade	Progressive	(Zobor *et al.*, 2022)
c.889C>T	p.(Arg256>Ter)	4	03	Pakistani	Yes	Yes	First Decade	Progressive	(Wang, 2019)
c.2218-2219insT	p.(Tyr700fsX714)	17	04	Pakistani	Yes	Yes	First Decade	Progressive	(Khan *et al.*, 2021)
c.2028-1G>A	P.(Lys677Argfs*24)	16	06	Pakistani	Yes	Yes	First Decade	Progressive	(Khateb *et al.*, 2019)

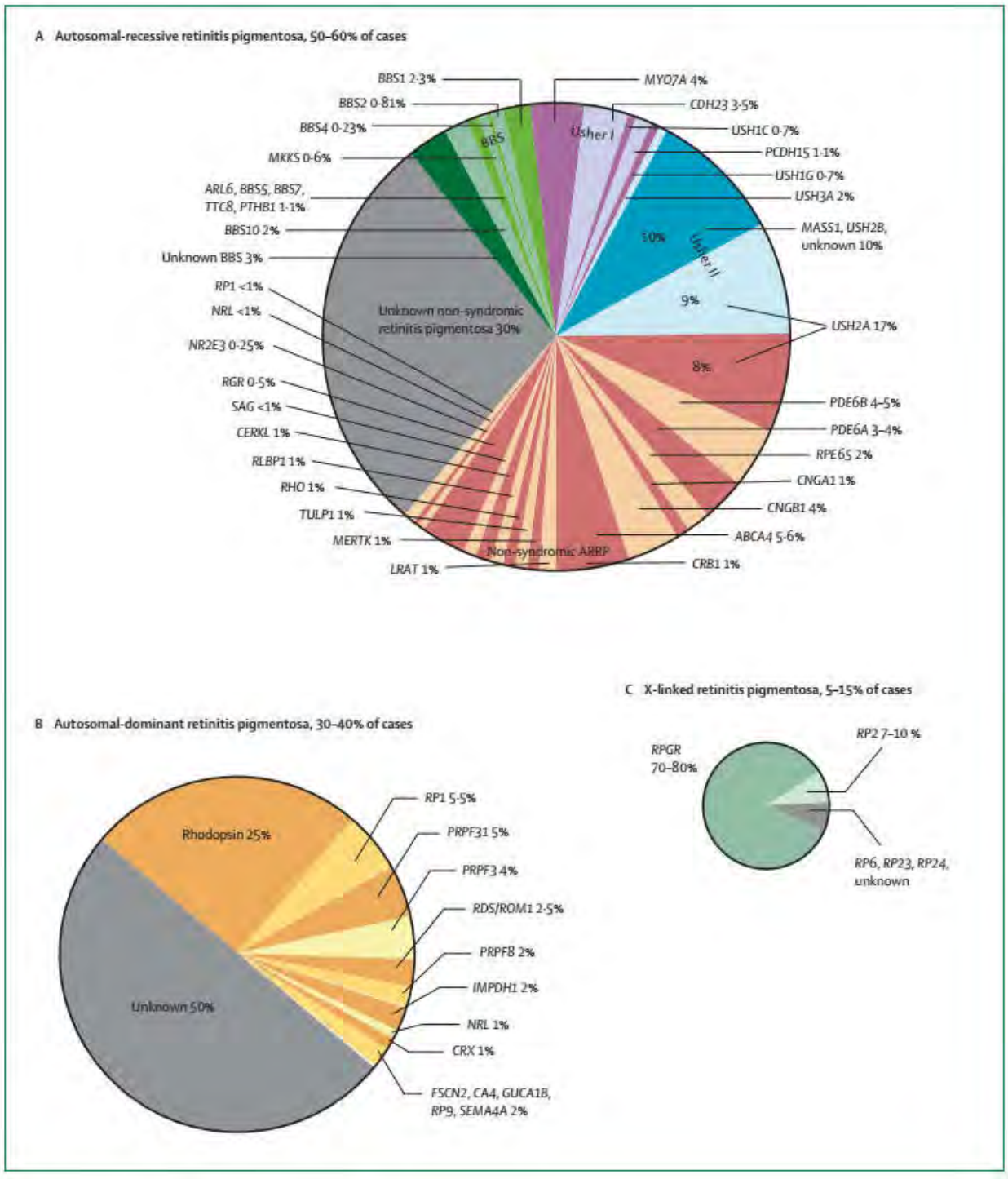


Fig. 2.0: Genes and their relative contribution to retinitis pigmentosa (Hartong *et al.*, 2006)

1.5. Objectives:

Objectives of the current study are:

- To enroll clinically diagnosed Retinitis pigmentosa cases having positive family history.
- To find relative incidence of Syndromic and Non-Syndromic Retinitis pigmentosa.
- To screen prevalent mutation c.304C>A, p.Arg102Ser of *PDE6A* gene in all enrolled cases.

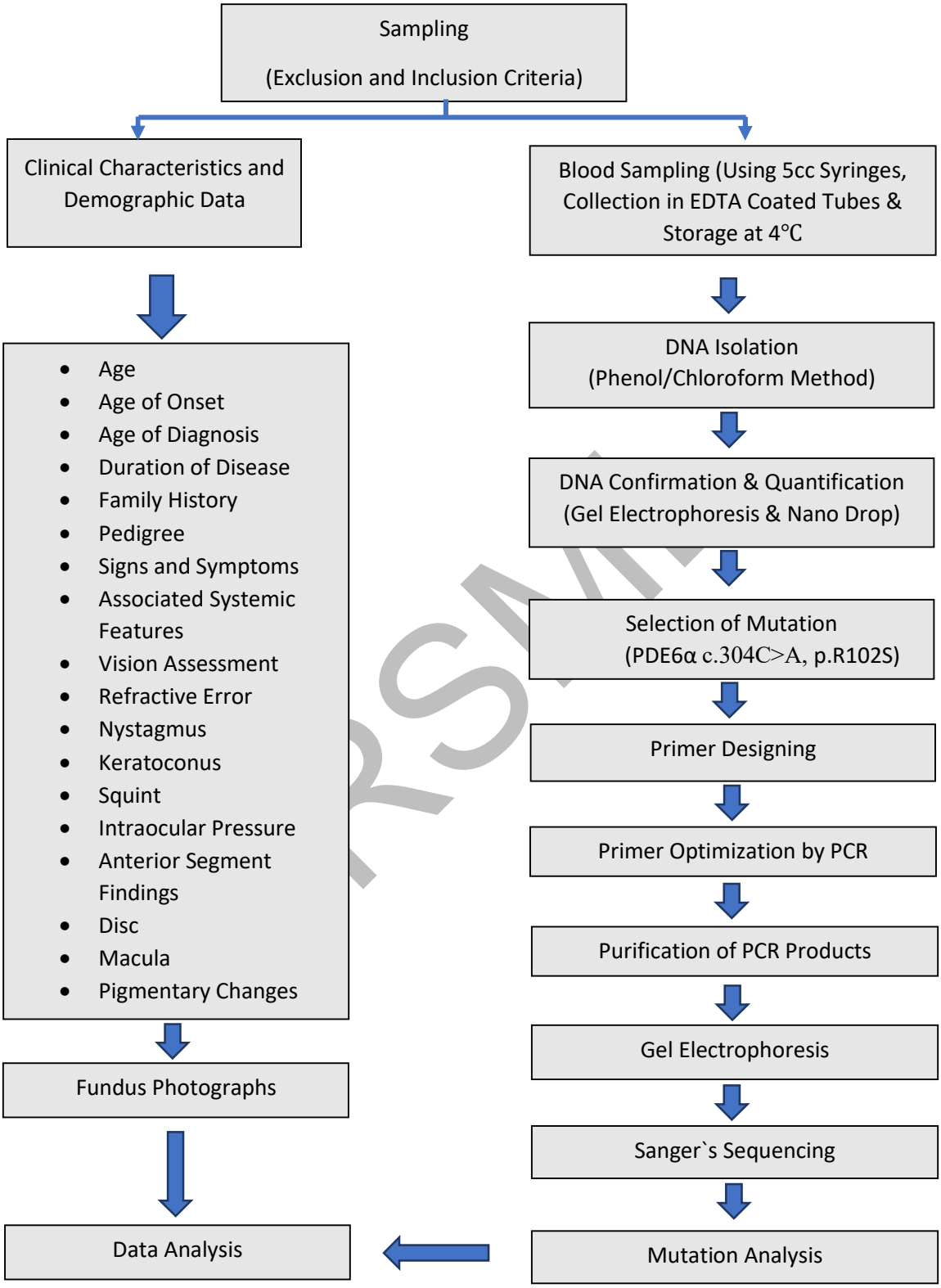


Fig. 2.1: Steps of Methodology

Chapter No. 2:

MATERIALS AND METHODS

2.1. Ethical Approval:

The current study was approved by the Bioethical review committee, Department of Zoology, Faculty of Biological Sciences, Quaid-i-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 included in the study were from different regions of Pakistan.

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. HaploPainter1.043 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 the Retinitis Pigmentosa patients. The diagonal line on the symbols represents the deceased individuals. The double lines between the two symbols shows the cousin marriage. To represent different generations, Roman numerals were used while the individuals in each generation were denote by Arabic numerals.

2.4. Blood Collection:

Sampling of the families from Bannu was carried out at Khalifa Gul Nawaz Hospital, Bannu, KPK and District Headquarter Hospital Bannu, KPK and of those who were from other 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 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 exclusion 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:

To extract the genomic DNA, an organic extraction method of genomic DNA Extraction i.e. Phenol-Chloroform Method was used. The protocol of genomic DNA isolation is given below.

A. 1st Day of DNA Extraction:

- EDTA coated tubes having the 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 2mM 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 20-25 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 the 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.

B. 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℃.
- 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℃ and 3000 rpm.
- After centrifugation, three layers was visualized. The upper layer contains 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 became visible by precipitation.
- Falcon tubes were left for 5 to 10 minutes at room temperature.
- Tubes were centrifuged for 20 minutes at 20℃ and 3000 rpm and DNA pallet was formed.
- After centrifuge, supernatant was discarded very carefully, leaving the DNA pellet adhered to the wall of tubes.
- DNA pallets were washed with ethanol by adding 5 ml of chilled 70% Ethanol.
- Tubes were centrifuged for 20 minutes at 20℃ and 3000 rpm.
- After centrifuge, supernatant was discarded very carefully, leaving the DNA pellet adhered to the wall of tubes.
- The DNA pellet was dried by keeping the falcon tubes inverted on tissue paper for 2 to 3 hours.

- The pellet was dissolved in TE Buffer (according to the quantity of DNA threads), vortexed and placed in incubator for overnight at 37℃ to dissolve the DNA in buffer.

C. 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℃ 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℃ in properly labelled cryobox.

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

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

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 0.5gm 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. While the 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 (Dawlance) 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 the 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 prepared 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	Compositions
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-claved filtered water (50 ml) Ethidium Bromide (0.5 g)
6	**Loading Dye (25 ml)**	Auto-claved filtered water (25 ml) Bromo- phenol blue (0.0875 g) 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:

GTGTCAGATATTTAAGAAAACTAACAGAGGTCAGAGAAGACACACCTACAGC

AAGTAGACTGTCCCTGTGCTGCCTTTTTGCAACCCCTGCTTTGGCAGTGCTCA

AGCCCACCTCCTGCTCTGTGCAGACATCTCTTCTTTGCTCTTACTAGACCAAG

GTGAAAGAAAACTCTCACCTTCTCCCATCTGGCCCCACAGCATCTGGAACAC

ACTGATCCTCATAATCCTTGTTCTTGAGAAATATTAATGACTTAATCTCCCAA

GCTTGCTCCCTCTCCTGTGCAGGCCATCTCAGTATGTTTTGCAGACAAGACCC

AGAGAAGTCCAGACTGGACTTGTTGCAGACTGCAAAACTGCCATTGGAAGGC

CTCCGTCCCAGTCCTTCTACAGAGTAGCCAGTGGGATTCCCAGCCATGGGCG

AGGTGACAGCAGAGGAGGTGGAGAAGTTCCTGGACTCGAATATTGGCTTTGC

CAAACAGTACTACAACCTCCACTACCGGGCCAAGCTCATCTCCGACCTCCTTG

GGGCCAAGGAGGCTGCCGTGGACTTCAGCAACTACCACTCCCCGAGCAGCAT

GGAGGAGAGCGAAATCATCTTTGATCTCCTGCGGGACTTTCAGGAGAATTTA

CAGACAGAGAAATGCATCTTCAATGTCATGAAGAAGCTGTGCTTCCTCCTGC

AGGCAGACCGCATGAGCCTGTTCATGTACCGGACCCGCAATGGCATCGCAGA

GCTGGCCACCAGGCTTTTCAATGTCCACAAGGATGCTGTCCTCGAGGACTGC

CTGGTGATGCCCGACCAAGAGATCGTCTTCCCTTTGGACATGGGCATCGTGG

GCCATGTCGCACACTCTAAGAAGATTGCTAACGTCCCCAACACAGAGGAGGT

ACTCTCTTCCCCATGAGAGAGAGGGCATGGGGCATTATTACATGGAGTTCTG

GGGTACAGGTGGGGTGAGGGGCATTGGCCACCAAGACAAGGCTGGTGACAA

CATGGTGCTTCTTCTTTATTTATTTACATATTTACTCATAATTCTTTATTTTGAA

AAAAAAATTCAAACTTAACAGAAAAGTTTGAAATAATACAGAGAACTGTATG

TCTTTCATCCAAATTTATCATTCATTAACATCTT

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 of 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 2.2.

Table 2.3.: Primers for Selected Mutation

Gene	Exon	Locus	Primer	Sequence (5`→3`)	Primer Length (bp)	Product Size (bp)	T_M
PDE6α	1	PDE6α c.304C>A, p.R102S Mutation	Forward Primer	5`-ACTACCGGGCCAAGCTCATCTCCGACCT-3`	28	367	65 °C
			Reverse Primer	5`-TTCTTAGAGTGTGCGACATGGCCCACGA-3`	28		

2.13. Primer Dilution:

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

2.14. Polymerase Chain Reaction:

The selected exon (Exon 1) was amplified by using polymerase chain reaction (PCR) in all the member of 25 families. PCR was performed in PCR tubes (Axygen,

USA) having 200 µl capacity. The chemicals (Thermo-Scientific PCR Kit) and their volume used in this reaction mixture are given in table 2.3.

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	2.5 µl
2	dNTPs	2.5 mM	2.5 µl
3	$MgCl_2$	25 mM	2 µl
4	Forward Primer	10 pmol/µl	0.5 µl
5	Reverse Primer	10 pmol/µl	0.5 µl
6	DNA	>100 ng/µl	2 µl
7	Taq Polymerase	5 U/µl	0.5 µl
8	PCR Water		14.5 µl
	Total Volume		**25 µl**

The PCR tubes were subjected for short spin in microfuge at 3000 rpm for 1 minute to mix the contents before placing them in thermos cycler (Bio-Rad T100) for PCR reaction. The thermal conditions set for PCR reaction are given in table 2.4.

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	65°C	45 sec	
Extension	72°C	60 sec	
Final Extension	72°C	10 min	1X
Hold	25°C	∞	

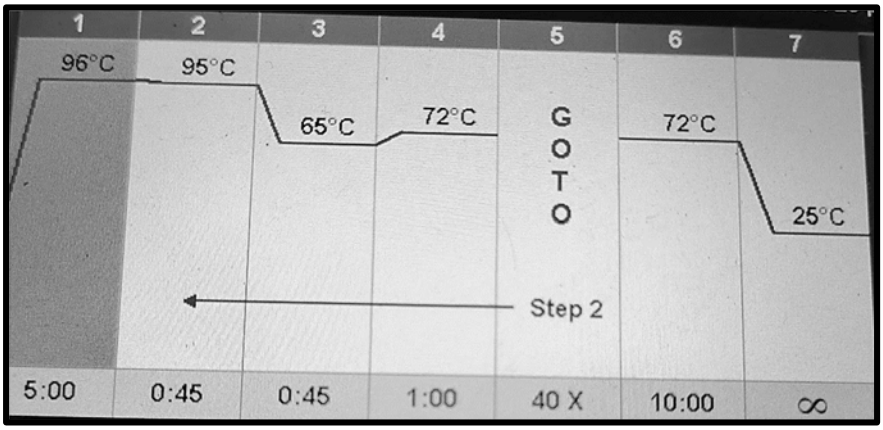


Fig. 2.2: Conditions for PCR Cycles

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 (Bromophenole 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.

i. An equal volume of binding buffer was added to PCR product.
ii. After tapping the tubes, the mixture was left for 1 minute at room temperature.
iii. Tubes were centrifuged at 13000 rpm for 2 minutes to mix it well.
iv. Samples were transferred to pre-labelled spin column tubes with collection tube attached.
v. Spin column tubes were centrifuged at 13000 rpm for 1 minute.
vi. 350 µl washing buffer was added in each sample and again centrifuged at 13000 rpm for 1 minute.
vii. Samples were kept at room temperature for 2 minutes.

viii. 25 µl Elution buffer was added in each sample. Elusion buffer was kept in incubator at 70°C before use.

ix. The spin column tubes were placed in pre-labelled Eppendorf tubes.

x. The samples were kept at room temperature for 2 minutes.

xi. Tubes were centrifuged at 13000rpm for 1 minute.

xii. 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 Sanger`s Sequencing to HEJ (Hussein Ebrahim Jamal) Research Institute, International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan under the grant by PCSIR (Pakistan Council of Scientific & Industrial Research, Islamabad, Pakistan), project titled as "Access to Scientific Instrumentation Programme - 2022". For sequencing, 8 µl of purified PCR product of each sample was mixed with the 4 µl of forward primer. Each tube was sealed with care and was sent for Sanger`s sequencing. Big Dye Terminator Chemistry (an automated ABI PRISM® 3730 Genetic Analyzer) was applied to accomplish the sequencing reaction. The labelled fragments of DNA were separated by capillary electrophoresis method and were detected by spectrum analyzer. Specific dye was used for the labelling of each nucleotide (Adenine, Thymine, Guanine and Cytosine) for documentation.

2.18. Mutation Analysis:

The sequenced data was aligned against the reference sequence taken from Ensemble genome browser (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://bio.tools/i-mutant), PROVEAN (http://provean.jcvi.org/index.php) and gnomAD (https://gnomad.broadinstitute.org/) were also used for novel and previously reported variants. Uniprot (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

(http://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.

Chapter No. 3:

RESULTS

3.1. Clinical Characteristics:

Blood samples of twenty-four families belonging to different regions of Pakistan were collected at Al-Shifa Eye Trust Hospital, Rawalpindi, Khalifa Gul Nawaz Hospital, Bannu and District Headquarter Hospital Bannu. 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 24 families, 11 were syndromic RP while 13 were Non-Syndromic RP families. Total individuals from which the blood samples were collected were 66 including 43 males and 23 females. Among them, 32 individuals were controls and 34 individuals were diagnosed with RP including 26 males and 8 females. The ratio of male RP patients was greater than the female RP patients i.e. 76.50% males and 23.50% females. The mean age of all the probands was ±23.54 years included the mean age of male proband was ±23.05 years and of female proband was ±25.40 years. The mean age of onset of RP was ±2.16 years while the mean age of diagnosis of RP was ±2.97 years.

From each family, proband was selected for mutation analysis of c.304C>A, p.R102S mutation in exon 1 of *PDE6A* gene which is associated with RP. 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 the fundus examination of all the affected individuals. The proband of each family had typical fundus observation including boney spicules, attenuated arteries, pigmentation, and waxy pallor disc.

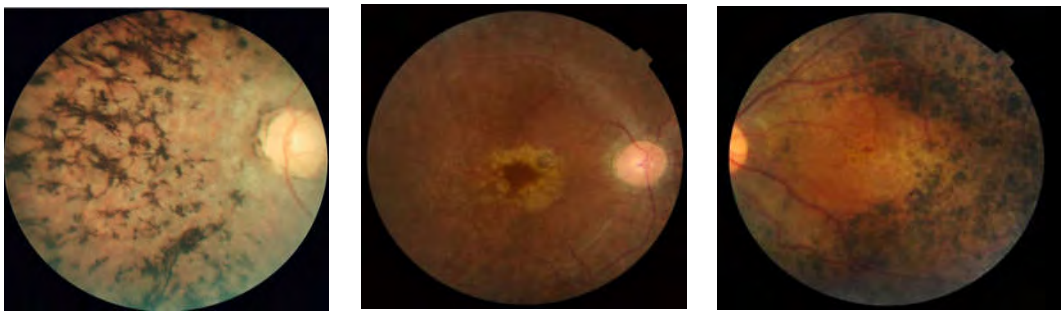
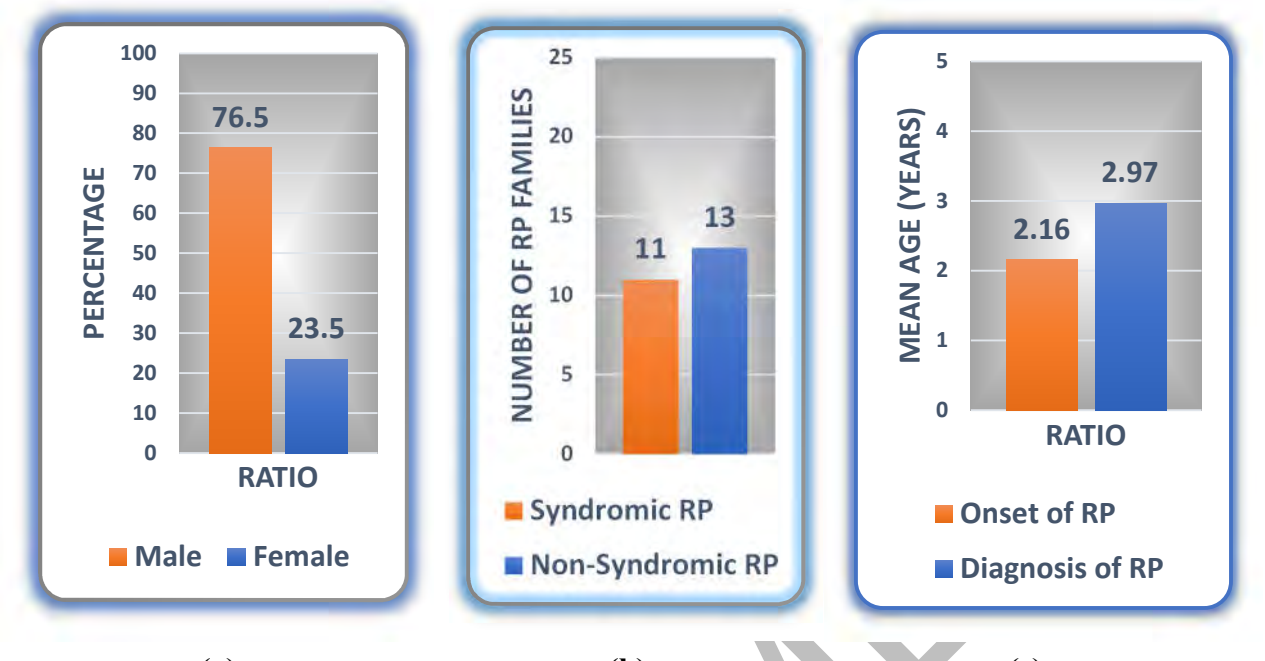


Fig. 3.1: Representative fundus photographs of RP patients.



(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 (11) and Non-Syndromic (13) RP families. (c) Graph representing the mean values of onset of RP and mean value of diagnosis of RP in collected families.

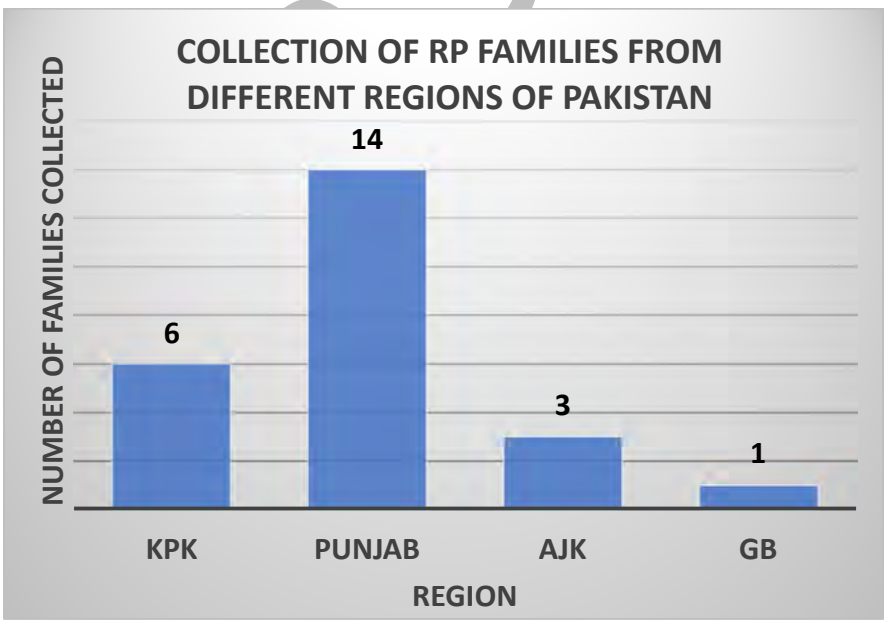


Fig. 3.3: Graph representing number of RP families from different regions of Pakistan.

Table 3.1: Clinical Findings of Families having Retinitis Pigmentosa

Sr. No.	Family I.D	Ethnicity	No. of Effected Persons in Family	Proband	Sex	Family History	Clinical Parameters						
							Syndromic/Non-Syndromic RP	Age of Onset	Progression	I.O.P	Visual Acuity		Other Findings
1	RP117-P1	Pathan	02	IV-2	F	✓	NS-RP	2 Y	Progressive	Normal	6/18	6/18	MD, P, POD, AA
2	RP118-P1	Pathan	04	IV-2	M	✓	NS-RP	3 Y	Progressive	Normal	6/24	6/38	MD, P, POD, AA
3	RP119-P1	Butt	03	IV-1	M	✓	S-RP	1 Y	Progressive	Normal	6/36	6/40	MD, P, POD, AA, MR
4	RP120-P1	Rajpoot	03	IV-1	M	✓	S-RP	2 Y	Progressive	Normal	6/24	6/30	MD, P, POD, AA, IS, HL
5	RP121-P1	Pathan	04	IV-3	M	✓	S-RP	3 Y	Progressive	Normal	6/24	6/15	MD, P, POD, AA
6	RP122-P1	Pathan	04	IV-1	M	✓	NS-RP	2 Y	Progressive	Normal	6/18	6/18	MD, P, POD, AA
7	RP123-P1	Mir	05	IV-5	F	✓	NS-RP	3 Y	Progressive	Normal	6/24	6/20	MD, P, POD, AA, Epilepsy
8	RP124-P1	Tanoli	03	IV-4	M	✓	NS-RP	2 Y	Progressive	Normal	6/18	6/24	MD, P, POD, AA
9	RP125-P1	Rajpoot	04	IV-1	F	✓	NS-RP	1 Y	Progressive	Normal	6/24	6/15	MD, P, POD, AA, CB
10	RP126-P1	Gujjar	03	IV-2	M	✓	S-RP	1 Y	Progressive	Normal	6/20	6/30	MD, P, POD, AA, MR
11	RP127-P1	Rajpoot	04	IV-3	M	✓	NS-RP	2 Y	Progressive	Normal	6/18	6/15	MD, P, POD, AA
12	RP128-P1	Gujjar	04	IV-1	F	✓	NS-RP	2 Y	Progressive	Normal	6/18	6/96	MD, P, POD, AA
13	RP129-P1	Abbasi	03	IV-1	M	✓	NS-RP	3 Y	Progressive	Normal	6/46	6/60	MD, P, POD, AA
14	RP130-P1	Pathan	07	IV-1	M	✓	NS-RP	2 Y	Progressive	Normal	6/24	6/19	MD, P, POD, AA
15	RP131-P1	Abbasi	04	IV-1	M	✓	S-RP	1 Y	Progressive	Normal	6/30	6/60	MD, P, POD, AA, PD
16	RP132-P1	Koloch	03	IV-3	M	✓	S-RP	2 Y	Progressive	Normal	6/80	6/90	MD, P, POD, AA, IS
17	RP133-P1	Pathan	06	IV-2	F	✓	S-RP	1 Y	Progressive	Normal	6/40	6/36	MD, P, POD, AA, PD
18	RP134-P1	Pathan	04	IV-1	M	✓	NS-RP	4 Y	Progressive	Normal	6/38	6/30	MD, P, POD, AA
19	RP135-P1	Sardar	03	IV-1	M	✓	NS-RP	3 Y	Progressive	Normal	6/24	6/18	MD, P, POD, AA
20	RP136-P1	Bunsin	04	IV-2	M	✓	NS-RP	3 Y	Progressive	Normal	6/24	6/20	MD, P, POD, AA
21	RP137-P1	Rajpoot	02	IV-1	M	✓	S-RP	2 Y	Progressive	Normal	6/18	6/24	MD, P, POD, AA, HT, HL
22	RP138-P1	Rajpoot	03	IV-1	M	✓	S-RP	2 Y	Progressive	Normal	6/24	6/19	MD, P, POD, AA, HT, HL
23	RP139-P1	Rajpoot	03	IV-1	M	✓	S-RP	2 Y	Progressive	Normal	6/30	6/60	MD, P, POD, AA, PD
24	RP140-P1	Minhas	03	IV-1	M	✓	S-RP	3 Y	Progressive	Normal	6/18	6/15	MD, P, POD, AA, Low I.Q

Key: M=Male, F=Female, Y=Year, S-RP=Syndromic RP, NS-RP=NON-Syndromic RP, MD=Macular Degeneration, P=Pigmentation, POD=Pale Optic Disc, AA=Attenuated Arteries, MR=Mental Retardation, HT=Hypertension, HL=Hearing Loss, CB=Colour Blindness, IS=Impaired Speech, PD=Polydactyly

3.1.1. Family RP-117:

The family RP-117 belongs to Bannu, Khyber Pakhtunkhwa, Pakistan. This family consisted of 18 members having two affected members, both were females (IV-2 and IV-4). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 24 years and 5 years respectively. All the affected members of 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 3.4 (A) representing the phenotypic expression and relationship of other members with proband.

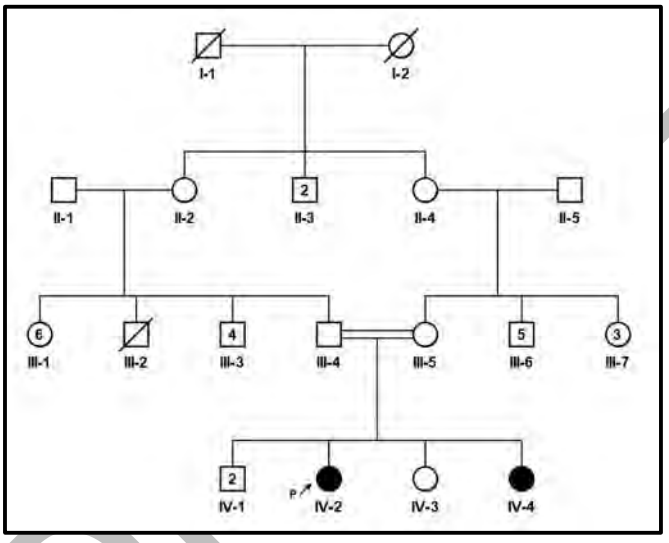


Fig. 3.4. (A): Pedigree of RP-117 Family.

3.1.2. Family RP-118:

The family RP-118 belongs to Bannu, Khyber Pakhtunkhwa, Pakistan. This family consisted of 41 members having four affected members, three were males (IV-2, IV-5 and IV-7) and one was female (IV-4). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 36, 22, 34 and 30 years respectively. All the affected members of 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 3.4 (B) representing the phenotypic expression and relationship of other members with proband.

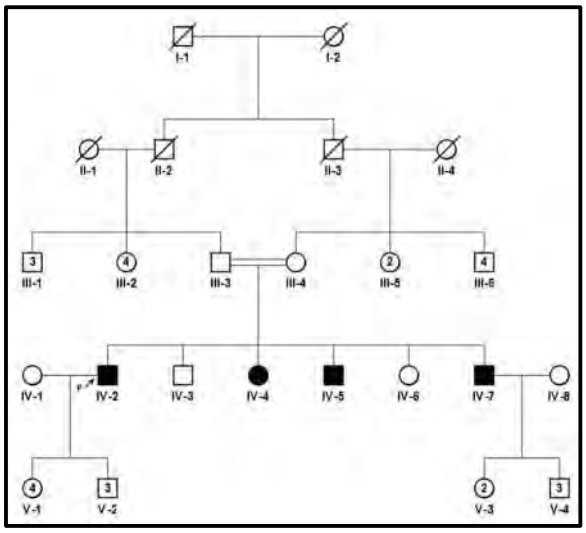


Fig. 3.4. (B): Pedigree of RP-118 Family.

3.1.3. Family RP-119:

The family RP-119 belongs to Jhelum, Punjab, Pakistan. This family consisted of 16 members having three affected members, all three were males (III-6, IV-1 and IV-2). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 28, 23 and 25 years respectively. All the affected members of family had progressive night blindness, visual impairment, bilateral RP and mental retardation. This family had Syndromic RP. The pedigree shows the pattern of RP as autosomal recessive. Figure 3.4 (C) representing the phenotypic expression and relationship of other members with proband.

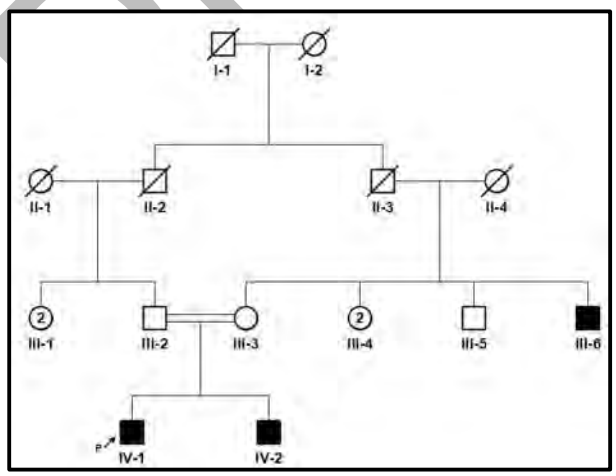


Fig. 3.4. (C): Pedigree of RP-119 Family.

3.1.4. Family RP-120:

The family RP-120 belongs to Jhelum, Punjab, Pakistan. This family consisted of 18 members having three affected members, two were males (IV-1 and IV-3) and one was female (III-3). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 24, 16 and 60 years respectively. All the affected members of family had progressive night blindness, visual impairment, bilateral RP, impaired speech, and hearing loss. This family had Syndromic RP. The pedigree shows the pattern of RP as autosomal recessive. Figure 3.4 (D) representing the phenotypic expression and relationship of other members with proband.

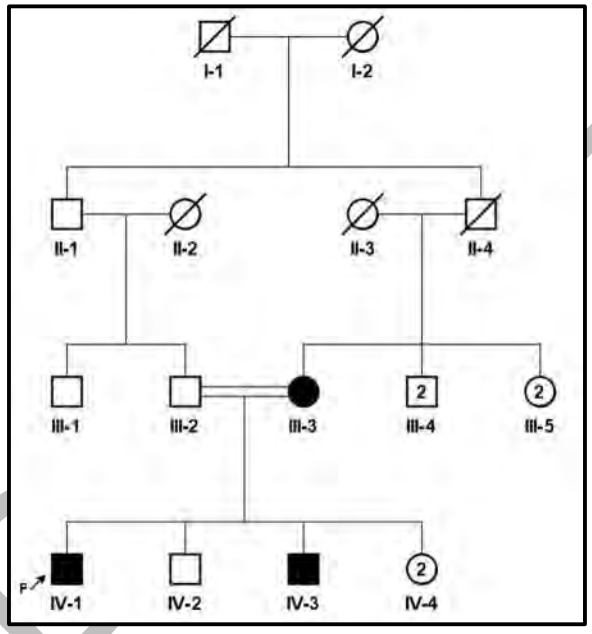


Fig. 3.4. (D): Pedigree of RP-120 Family.

3.1.5. Family RP-121:

The family RP-121 belongs to Rawalpindi, Punjab, Pakistan. This family consisted of 34 members having four affected members, two were males (IV-3 and IV-6) and two were females (IV-2 and IV-7). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 21, 24, 25 and 31 years respectively. All the affected members of 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 3.4 (E) representing the phenotypic expression and relationship of other members with proband.

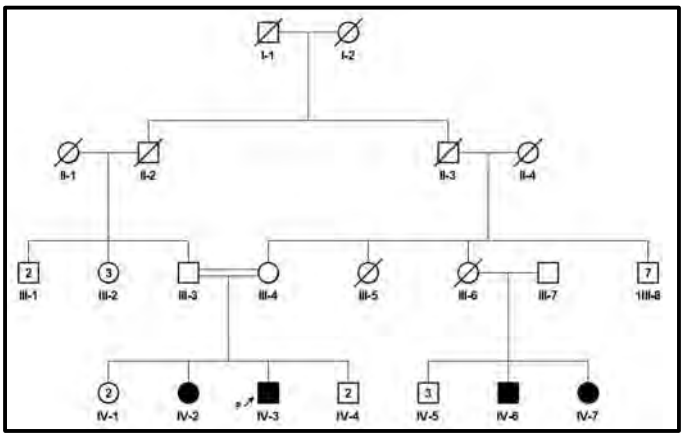


Fig. 3.4. (E): Pedigree of RP-121 Family.

3.1.6. Family RP-122:

The family RP-122 belongs to Haripur, Khyber Pakhtunkhwa, Pakistan. This family consisted of 29 members having four affected members, three were males (IV-1, IV-4 and IV-5) and one was female (IV-6). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 12, 19, 17 and 19 years respectively. All the affected members of 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 3.4 (F) representing the phenotypic expression and relationship of other members with proband.

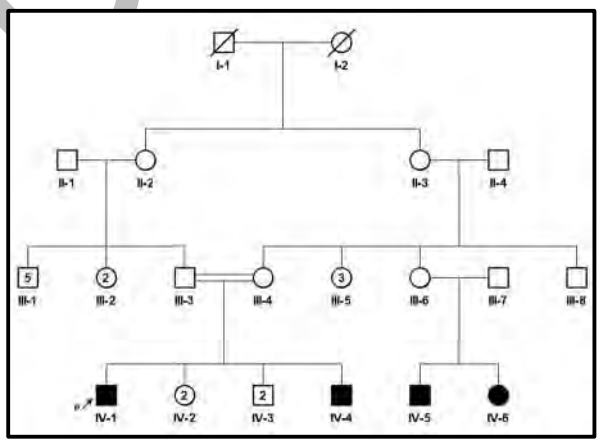


Fig. 3.4. (F): Pedigree of RP-122 Family.

3.1.7. Family RP-123:

The family RP-123 belongs to Poonch, Azad Jammu and Kashmir, Pakistan. This family consisted of 40 members having five affected members, four were females (IV-1, IV-3, IV-5 and V-3) and one was male (V-4). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 37, 29, 35, 17 and 15 years respectively. All the affected members of family had progressive night blindness, visual impairment, and bilateral RP. Individual IV-5 had epilepsy condition along with RP. This family had Non-Syndromic RP. The pedigree shows the pattern of RP as autosomal recessive. Figure 3.4 (G) representing the phenotypic expression and relationship of other members with proband.

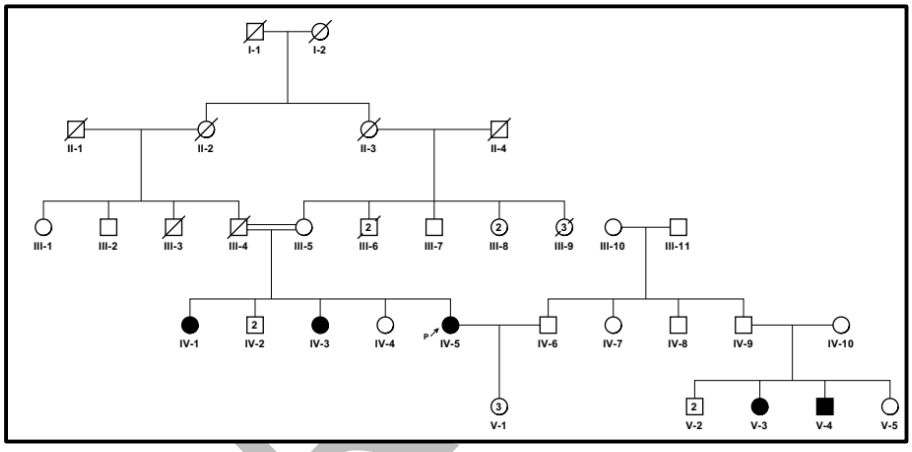


Fig. 3.4. (G): Pedigree of RP-123 Family.

3.1.8. Family RP-124:

The family RP-124 belongs to Rawalpindi, Punjab, Pakistan. This family consisted of 20 members having three affected members, two were males (IV-4 and IV-5) and one was female (IV-2). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 14, 11 and 21 years respectively. All the affected members of 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 3.4 (H) representing the phenotypic expression and relationship of other members with proband.

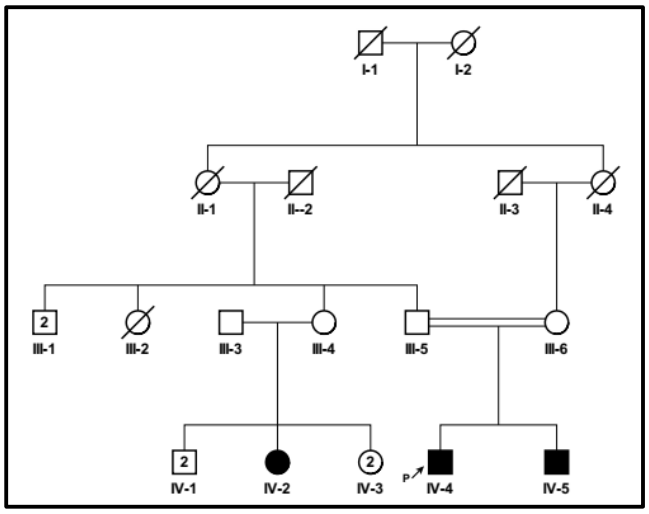


Fig. 3.4. (H): Pedigree of RP-124 Family.

3.1.9. Family RP-125:

The family RP-125 belongs to Chakwal, Punjab, Pakistan. This family consisted of 25 members having four affected members, all four were males (III-2, III-4, IV-1 and V-1). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 68, 65, 25 and 7 years respectively. All the affected members of family had progressive night blindness, visual impairment, and bilateral RP. The proband also had colour blindness condition in addition to RP. This family had Syndromic RP. The pedigree shows the pattern of RP as autosomal recessive. Figure 3.4 (I) representing the phenotypic expression and relationship of other members with proband.

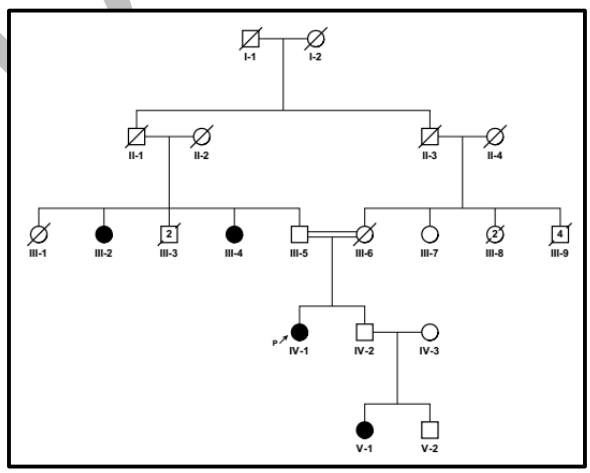


Fig. 3.4. (I): Pedigree of RP-125 Family.

3.1.10. Family RP-126:

The family RP-126 belongs to Jhelum, Punjab, Pakistan. This family consisted of 24 members having three affected members, two were males (IV-2 and IV-3) and one was female (III-7). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 16, 18 and 54 years respectively. All the affected members of family had progressive night blindness, visual impairment, and bilateral RP. The proband also had mental retardation condition in addition to RP. This family had Syndromic RP. The pedigree shows the pattern of RP as autosomal recessive. Figure 3.4 (J) representing the phenotypic expression and relationship of other members with proband.

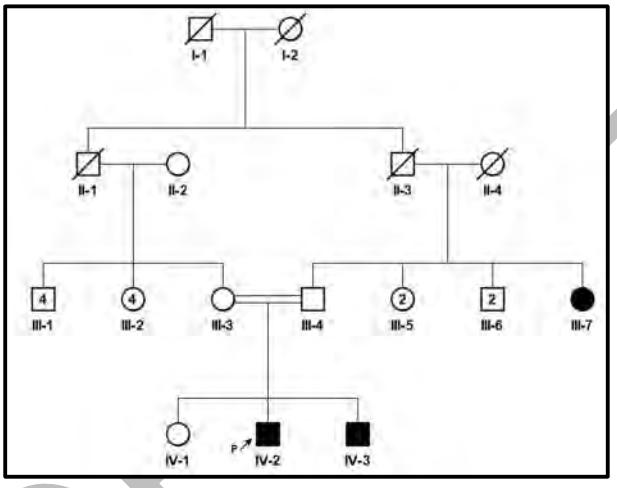


Fig. 3.4. (J): Pedigree of RP-126 Family.

3.1.11. Family RP-127:

The family RP-127 belongs to Bagh, Azad Jammu and Kashmir, Pakistan. This family consisted of 29 members having four affected members, three were females (II-3, III-4 and IV-4) and one was male (IV-3). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 90, 43, 34 and 30 years respectively. All the affected members of 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 3.4 (K) representing the phenotypic expression and relationship of other members with proband.

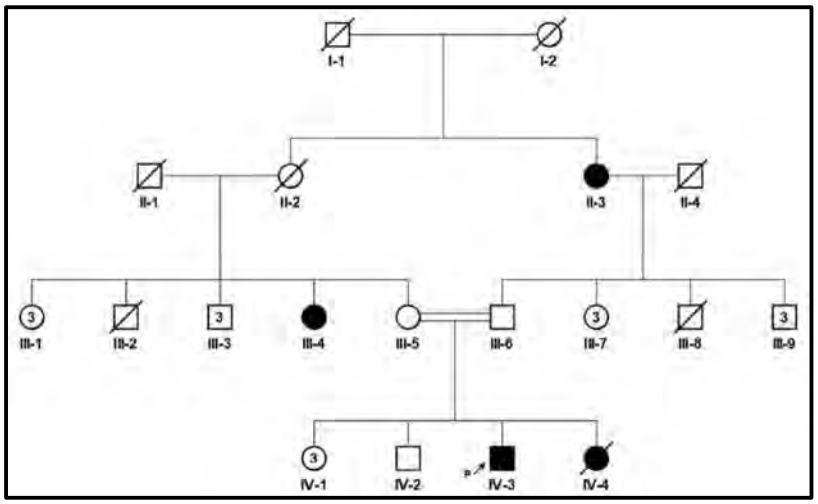


Fig. 3.4. (K): Pedigree of RP-127 Family.

3.1.12. Family RP-128:

The family RP-128 belongs to Gujrat, Punjab, Pakistan. This family consisted of 24 members having four affected members, three were females (II-4, IV-1 and IV-2) and one was male (III-1). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 70, 23, 21 and 43 years respectively. All the affected members of 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 3.4 (L) representing the phenotypic expression and relationship of other members with proband.

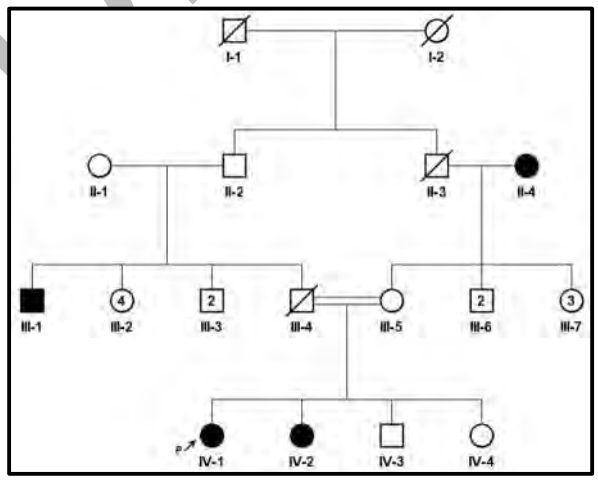


Fig. 3.4. (L): Pedigree of RP-128 Family.

3.1.13. Family RP-129:

The family RP-129 belongs to Rawalpindi, Punjab, Pakistan. This family consisted of 19 members having three affected members, two were males (II-1 and IV-1) and one was female (II-4). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 75, 12 and 72 years respectively. All the affected members of 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 3.4 (M) representing the phenotypic expression and relationship of other members with proband.

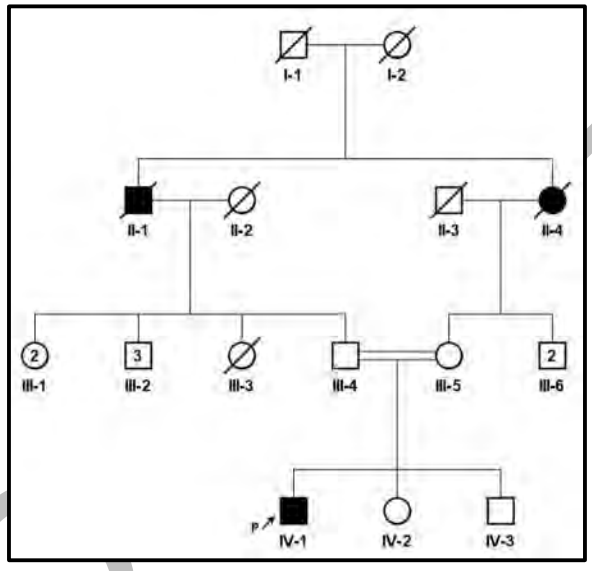


Fig. 3.4. (M): Pedigree of RP-129 Family.

3.1.14. Family RP-130:

The family RP-130 belongs to Swat, Khyber Pakhtunkhwa, Pakistan. This family consisted of 41 members having seven affected members, all seven were males (II-1, II-3, IV-1, IV-2, IV-6, IV-7 and IV-8). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 90, 87, 16, 30, 21, 35 and 28 years respectively. All the affected members of 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 3.4 (N) representing the phenotypic expression and relationship of other members with proband.

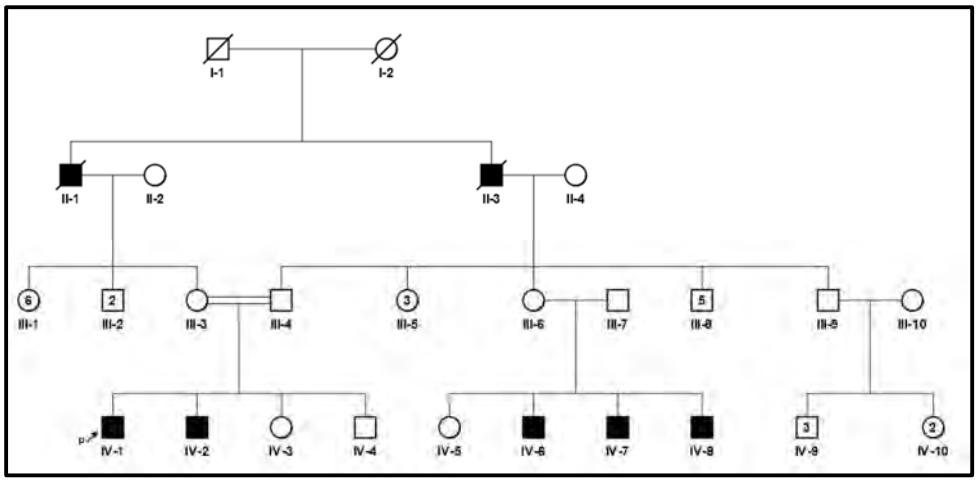


Fig. 3.4. (N): Pedigree of RP-130 Family.

3.1.15. Family RP-131:

The family RP-131 belongs to Sudhanoti, Azad Jammu and Kashmir, Pakistan. This family consisted of 21 members having four affected members, two were males (IV-1 and IV-3) and two were females (II-4 and IV-4). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 35, 30, 82 and 36 years respectively. All the affected members of family had progressive night blindness, visual impairment, and bilateral RP. The proband also had polydactyly condition along with RP. This family had Syndromic RP. The pedigree shows the pattern of RP as autosomal recessive. Figure 3.4 (O) representing the phenotypic expression and relationship of other members with proband.

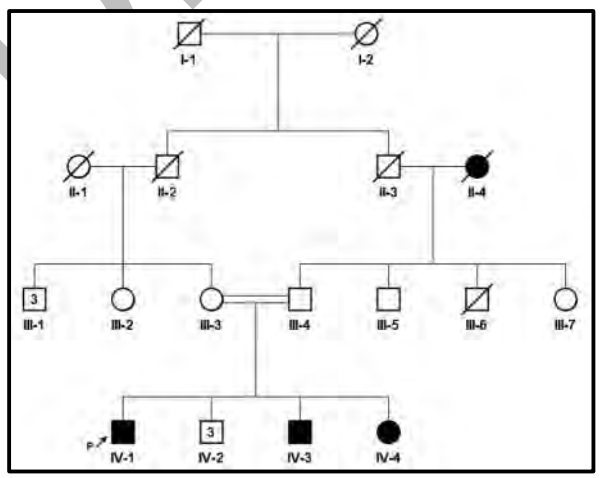


Fig. 3.4. (O): Pedigree of RP-131 Family.

3.1.16. Family RP-132:

The family RP-132 belongs to Día Mir, Gilgit Baltistan, Pakistan. This family consisted of 20 members having three affected members, two were males (III-4 and IV-3) and one was female (IV-4). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 18, 52 and 22 years respectively. All the affected members of family had progressive night blindness, visual impairment, and bilateral RP. All the affected individuals had speech impairment condition along with RP. This family had Syndromic RP. The pedigree shows the pattern of RP as autosomal recessive. Figure 3.4 (P) representing the phenotypic expression and relationship of other members with proband.

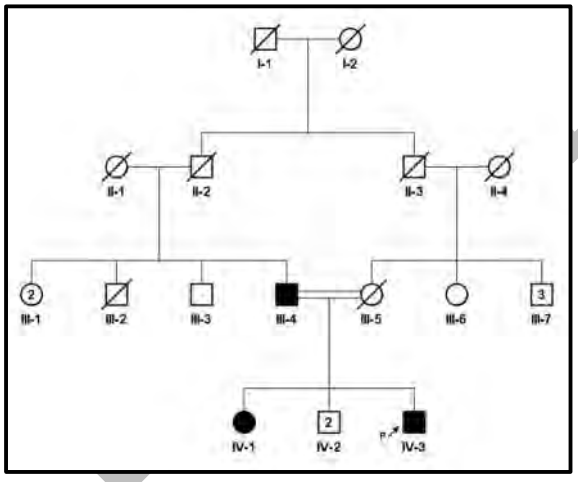


Fig. 3.4. (P): Pedigree of RP-132 Family.

3.1.17. Family RP-133:

The family RP-133 belongs to North Waziristan, Khyber Pakhtunkhwa, Pakistan. This family consisted of 34 members having six affected members, four were females (II-1, IV-2, IV-4 and IV-5) and two were males (III-6 and IV-6). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 72, 22, 10, 08, 49 and 06 years respectively. All the affected members of family had progressive night blindness, visual impairment, and bilateral RP. The proband also had feet disability condition along with RP. This family had Syndromic RP. The pedigree shows the pattern of RP as autosomal recessive. Figure 3.4 (Q) representing the phenotypic expression and relationship of other members with proband.

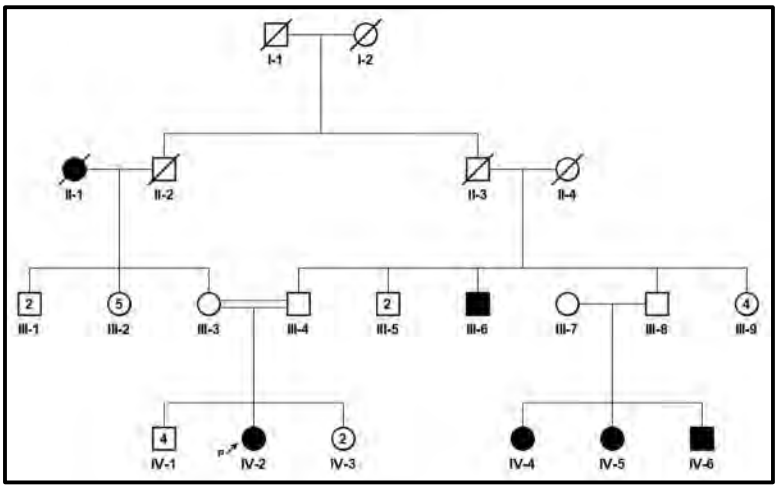


Fig. 3.4. (Q): Pedigree of RP-133 Family.

3.1.18. Family RP-134:

The family RP-134 belongs to Shangla, Khyber Pakhtunkhwa, Pakistan. This family consisted of 21 members having four affected members, two were males (IV-1 and IV-3) and two were females (III-3 and IV-5). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 20, 01, 35 and 16 years respectively. All the affected members of 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 3.4 (R) representing the phenotypic expression and relationship of other members with proband.

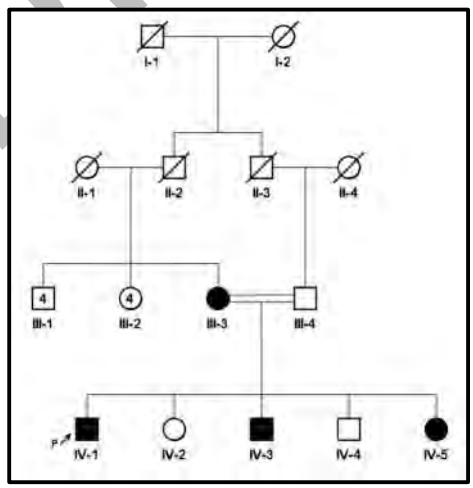


Fig. 3.4. (R): Pedigree of RP-134 Family.

3.1.19. Family RP-135:

The family RP-135 belongs to Rawalpindi, Punjab, Pakistan. This family consisted of 19 members having four affected members, three were males (II-3, IV-1 and IV-2) and one was female (III-5). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 72, 22, 18 and 42 years respectively. All the affected members of 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 3.4 (S) representing the phenotypic expression and relationship of other members with proband.

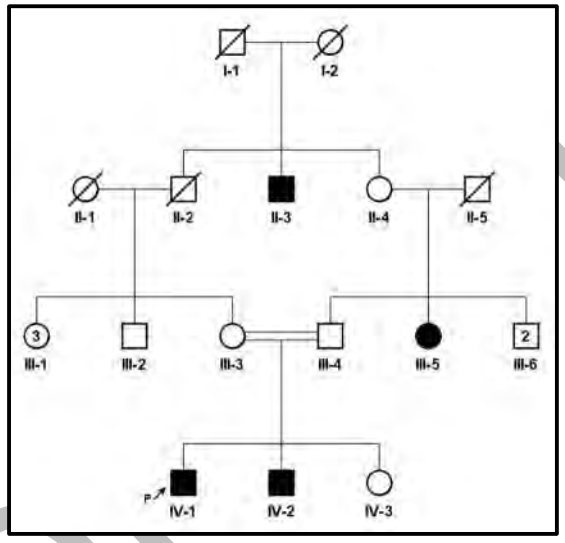


Fig. 3.4. (S): Pedigree of RP-135 Family.

3.1.20. Family RP-136:

The family RP-136 belongs to Mianwali, Punjab, Pakistan. This family consisted of 24 members having four affected members, three were males (III-7, IV-2 and IV-4) and one was female (IV-1). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 45, 21, 20 and 18 years respectively. All the affected members of 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 3.4 (T) representing the phenotypic expression and relationship of other members with proband.

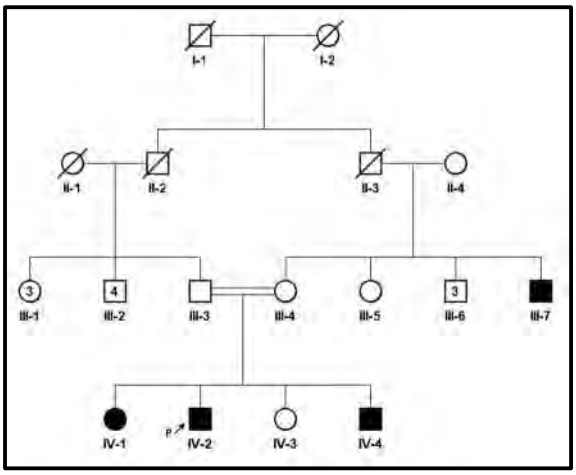


Fig. 3.4. (T): Pedigree of RP-136 Family.

3.1.21. Family RP-137:

The family RP-137 belongs to Rawalpindi, Punjab, Pakistan. This family consisted of 17 members having two affected members, one was male (IV-1) and one was female (II-1). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 33 and 72 years respectively. Both the affected members of family had progressive night blindness, visual impairment, bilateral RP, hearing loss and hypertension. This family had Syndromic RP. The pedigree shows the pattern of RP as autosomal recessive. Figure 3.4 (U) representing the phenotypic expression and relationship of other members with proband.

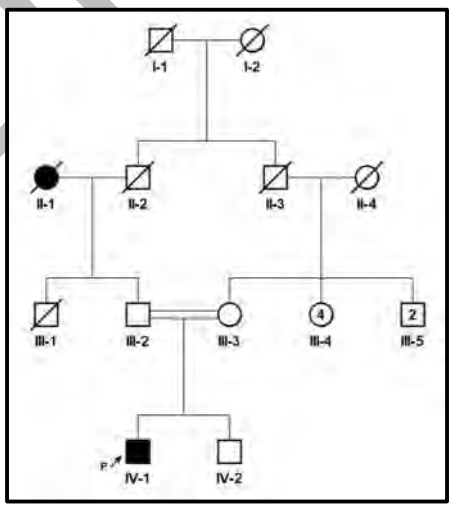


Fig. 3.4. (U): Pedigree of RP-137 Family.

3.1.22. Family RP-138:

The family RP-138 belongs to Rawalpindi, Punjab, Pakistan. This family consisted of 21 members having three affected members, two were males (III-6 and IV-1) and one was female (III-5). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 48, 35 and 55 years respectively. All the affected members of family had progressive night blindness, visual impairment, bilateral RP, hearing loss and hypertension. This family had Syndromic RP. The pedigree shows the pattern of RP as autosomal recessive. Figure 3.4 (V) representing the phenotypic expression and relationship of other members with proband.

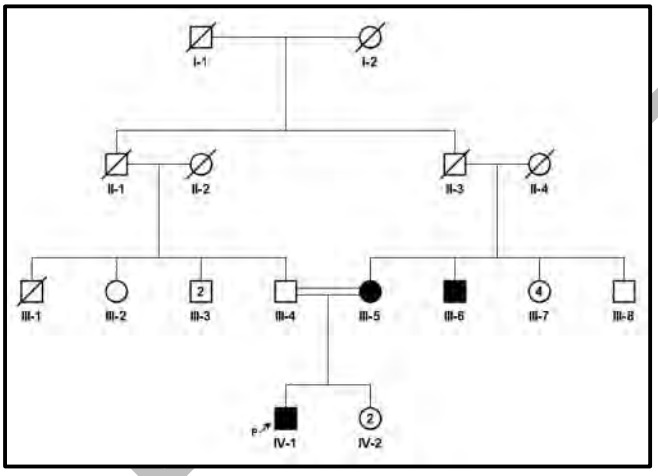


Fig. 3.4. (V): Pedigree of RP-138 Family.

3.1.23. Family RP-139:

The family RP-139 belongs to Rawalpindi, Punjab, Pakistan. This family consisted of 20 members having three affected members, all three were males (IV-2, IV-5 and IV-7). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 22, 25 and 33 years respectively. All the affected members of family had progressive night blindness, visual impairment, and bilateral RP. The proband also had polydactyly condition along with RP. This family had Syndromic RP. The pedigree shows the pattern of RP as autosomal recessive. Figure 3.4 (W) representing the phenotypic expression and relationship of other members with proband.

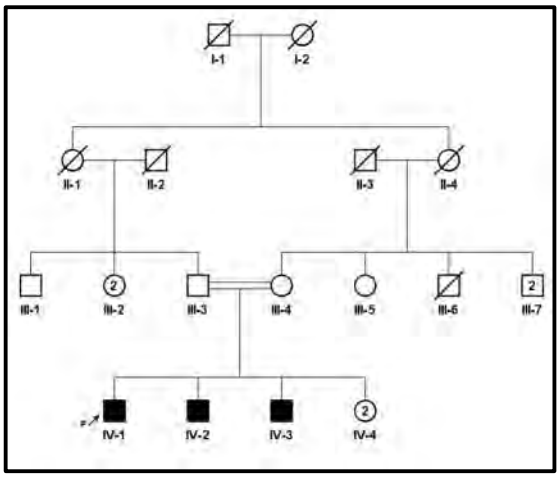


Fig. 3.4. (W): Pedigree of RP-139 Family.

3.1.24. Family RP-140:

The family RP-140 belongs to Rawalpindi, Punjab, Pakistan. This family consisted of 18 members having three affected members, all three were males (III-3, III-7 and IV-1). The descriptive data was collected from all alive members. The ages of affected individuals at the time of enrolment were 50, 47 and 35 years respectively. All the affected members of family had progressive night blindness, visual impairment, bilateral RP, hearing loss and low I.Q. This family had Syndromic RP. The pedigree shows the pattern of RP as autosomal recessive. Figure 3.4 (X) representing the phenotypic expression and relationship of other members with proband.

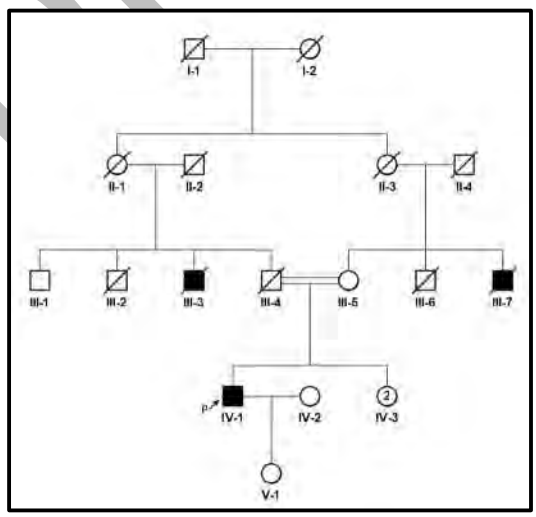


Fig. 3.4. (X): Pedigree of RP-140 Family.

3.2. DNA Isolation:

The DNA was isolated from each blood sample and the mean centration of the isolated DNA was up to 50 ng/µl with a purity value of 1.8 for each isolated sample.

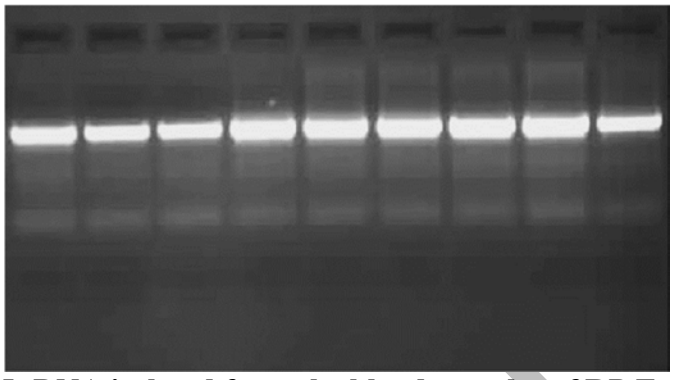


Fig. 3.5: DNA isolated from the blood samples of RP Families.

Table 3.2: Nano Drop Results of Retinitis Pigmentosa Families

Family No.	Member	Conc. (ng/µl)	A 260/280
Family No.1: RP117	RP117-C1	243.61	1.74
	RP117-C2	220.31	1.81
	RP117-C3	196.92	1.79
	RP117-C4	169.12	1.86
	RP117-P1	199.65	1.83
Family No.2: RP118	RP118-C1	174.26	1.76
	RP118-P1	185.54	1.76
	RP118-A1	129.85	1.78
	RP118-A2	119.46	1.76
Family No.3: RP119	RP119-C1	296.69	1.87
	RP119-P1	213.31	1.81
Family No.4: RP120	RP120-C1	219.69	1.86
	RP120-C2	177.66	1.85
	RP120-P1	185.17	1.78
Family No.5: RP121	RP121-C1	199.90	1.78
	RP121-P1	211.76	1.75
Family No.6: RP122	RP122-C1	126.92	1.77
	RP122-C2	189.19	1.82
	RP122-P1	171.31	1.84
	RP122-A1	151.56	1.82
	RP122-A2	163.89	1.83
	RP122-A3	149.81	1.79

Family No.	Member	Conc. (ng/µl)	A 260/280
Family No.7: RP123	RP123-C1	229.15	1.86
	RP123-P1	211.68	1.75
Family No.8: RP124	RP124-C1	125.18	1.73
	RP124-C2	155.49	1.75
	RP124-P1	194.04	1.85
Family No.9: RP125	RP125-C1	225.49	1.79
	RP125-P1	237.20	1.75
Family No.10: RP126	RP126-C1	288.00	1.79
	RP126-P1	198.57	1.82
	RP126-A1	199.81	1.81
Family No.11: RP127	RP127-C1	199.73	1.79
	RP127-P1	122.80	1.77
Family No.12: RP128	RP128-C1	457.03	1.78
	RP128-P1	186.58	1.83
	RP128-A1	122.59	1.69
Family No.13: RP129	RP129-C1	414.13	1.73
	RP129-P1	206.46	1.86
Family No.14: RP130	RP130-C1	318.28	1.69
	RP130-P1	306.07	1.82
	RP130-A1	333.00	1.79
Family No.15: RP131	RP131-C1	213.68	1.74
	RP131-P1	373.58	1.81
Family No.16: RP132	RP132-C1	186.28	1.65
	RP132-P1	140.07	1.72
Family No.17: RP133	RP133-C1	211.16	1.67
	RP133-P1	298.41	1.83
Family No.18: RP134	RP134-C1	277.21	1.76
	RP134-P1	253.63	1.74
	RP134-A1	419.10	1.69
Family No.19: RP135	RP135-C1	207.94	1.67
	RP135-P1	360.95	1.78
	RP135-A1	466.07	1.65
Family No.20: RP136	RP136-C1	211.87	1.66
	RP136-P1	188.90	1.81
Family No.21: RP137	RP137-C1	197.47	1.75
	RP137-P1	248.62	1.73
Family No.22: RP138	RP138-C1	201.32	1.69
	RP138-P1	248.56	1.69
Family No.23: RP139	RP139-C1	177.21	1.84
	RP139-P1	222.23	1.82

Family No.	Member	Conc. (ng/µl)	A 260/280
Family No.24: RP140	RP140-C1	149.95	1.73
	RP140-P1	176.22	1.71
Family No.25: RP141	RP141-C1	155.63	1.69
	RP141-P1	182.39	1.79
Average		**221.15**	**1.78**

3.3. Primer Optimization:

For optimization of primers gradient PCR was used. The annealing temperature, 65°C were found for primer pair designed for mutation analysis of c.304C>A, p.R102S mutation in exon 1 of *PDE6A* gene which is associated with RP.

3.4. Polymerase Chain Reaction:

Polymerase Chain Reaction (PCR) was performed to amplify the DNA samples of all the probands of collected families. The PCR products for c.304C>A, p.R102S mutation in exon 1 of *PDE6A* gene was of 367 bp were obtained by using gradient PCR. The confirmation of amplicon was done by running 2% of agarose gel electrophoresis and was observed in gel documentation system (Cleaver Scientific Limited) as shown in fig 3.6.

Fig. 3.6: In the first column 1kb DNA Marker was loaded and PCR products of 367bp in subsequent columns.

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 (Cleaver Scientific Limited). PCR amplicon of a family are shown in fig. 3.7.

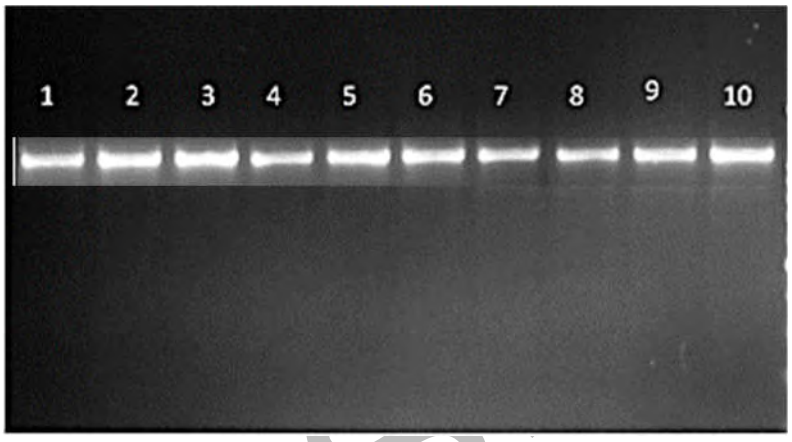


Fig. 3.7: Confirmation of Purified PCR products of 367bp.

3.6. Sanger`s Sequencing:

All the purified samples were sent for Sanger`s Sequencing to HEJ (Hussein Ebrahim Jamal) Research Institute, International Center for Chemical and Biological Sciences (ICCBS), University of Karachi, Pakistan under the grant by PCSIR (Pakistan Council of Scientific & Industrial Research, Islamabad, Pakistan), project titled as "Access to Scientific Instrumentation Programme - 2022".

Big Dye Terminator Chemistry (an automated ABI PRISM® 3730 Genetic Analyzer) was applied to accomplish the sequencing reaction. The labelled fragments of DNA were separated by capillary electrophoresis method and were detected by spectrum analyzer. Specific dye was used for the labelling of each nucleotide (Adenine, Thymine, Guanine and Cytosine) for documentation. Chromas 2.6.6 was used to open the Sanger`s sequencing results.

3.7. Genetic Analysis:

Mutations in *PDE6A* gene are known to cause RP in different populations globally. Exon 1 of *PDE6A* gene (Ensemble Transcript I.D: ENSE00003508743) having length of 594bp was selected to screen polymorphisms and mutations specially c.304C>A, p.R102S mutation in all enrolled RP families. *PDE6A* having Ensemble Transcript I.D: ENST00000255266.10 was used as a standard sequence for sequence alignment. All the analyzed sequences are listed below.

3.7.1. Family RP-117:

Sanger`s sequencing of exon 1 of RP117P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. However, one mutation (c.386A>G) was found in the sequence. The sequencing chromatogram is shown in fig 3.8.

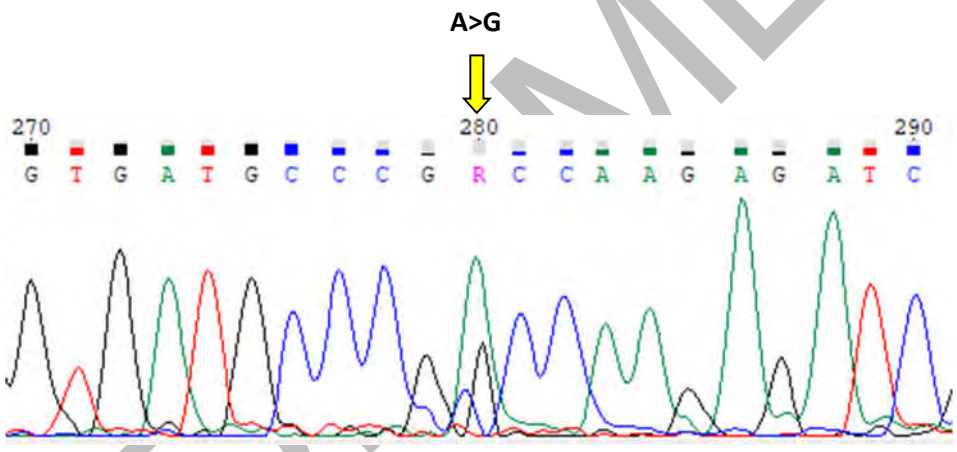


Fig. 3.8: Sequencing Chromatogram: Showing c.386A>G mutation at chr5:149323851T>C position in exon 1 of *PDE6A* gene of proband of RP-117 Family.

3.7.2. Family RP-118:

Sanger`s sequencing of exon 1 of RP118P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. Moreover, neither any polymorphism nor other mutation was found in the sequence. The sequencing chromatogram is shown in fig 3.9.

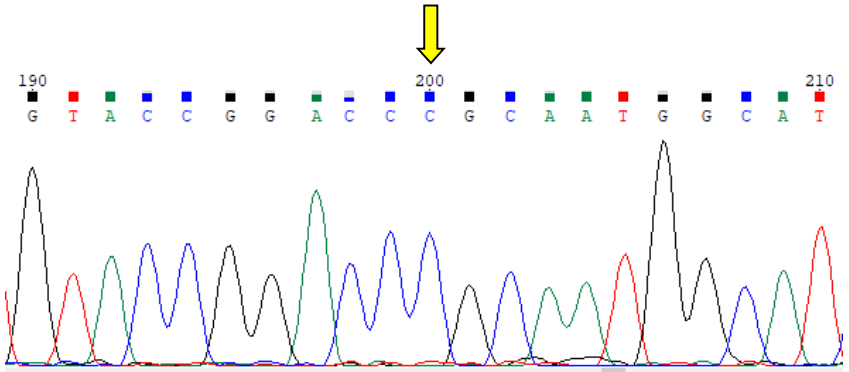


Fig. 3.9: Sequencing Chromatogram: Showing no mutation at chr5:149323933G>T position in exon 1 of *PDE6A* gene of proband of RP-118 Family.

3.7.3. Family RP-119:

Sanger`s sequencing of exon 1 of RP119P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. Moreover, neither any polymorphism nor other mutation was found in the sequence. The sequencing chromatogram is shown in fig 3.10.

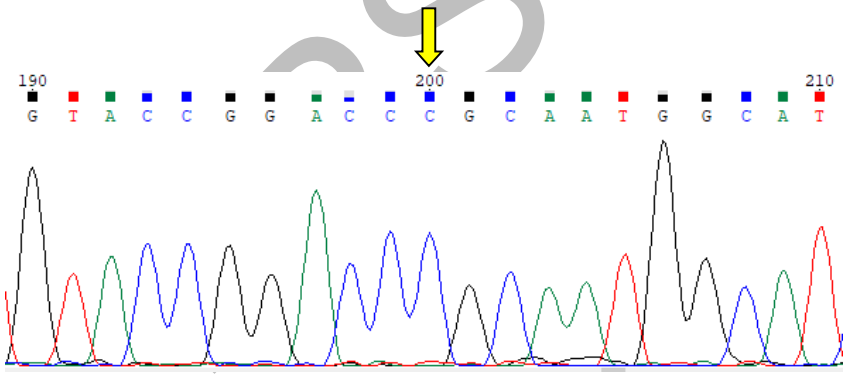


Fig. 3.10: Sequencing Chromatogram: Showing no mutation at chr5:149323933G>T position in exon 1 of *PDE6A* gene of proband of RP-119 Family.

3.7.4. Family RP-120:

Sanger`s sequencing of exon 1 of RP120P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. However, one polymorphism (c.331A>C) was found in the sequence. The sequencing chromatogram is shown in fig 3.11.

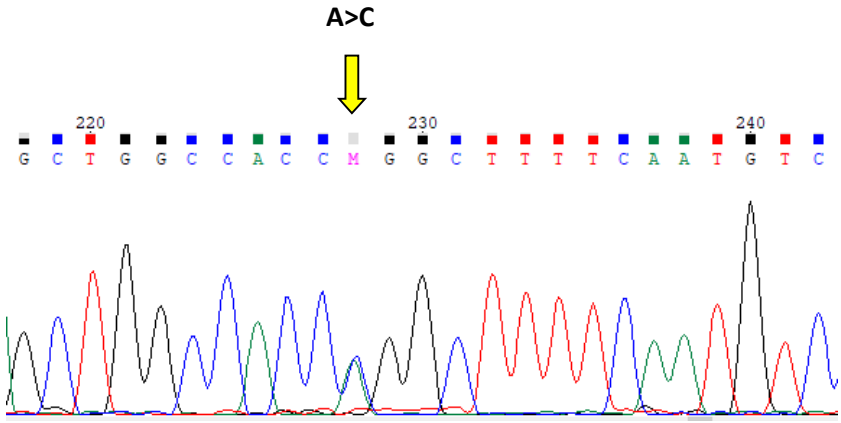


Fig. 3.11: Sequencing Chromatogram: Showing c.331A>C polymorphism (rs2277925) at chr5:149323906T>G position in exon 1 of *PDE6A* gene of proband of RP-120 Family.

3.7.5. Family RP-121:

Sanger`s sequencing of exon 1 of RP121P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. Moreover, neither any polymorphism nor other mutation was found in the sequence. The sequencing chromatogram is shown in fig 3.12.

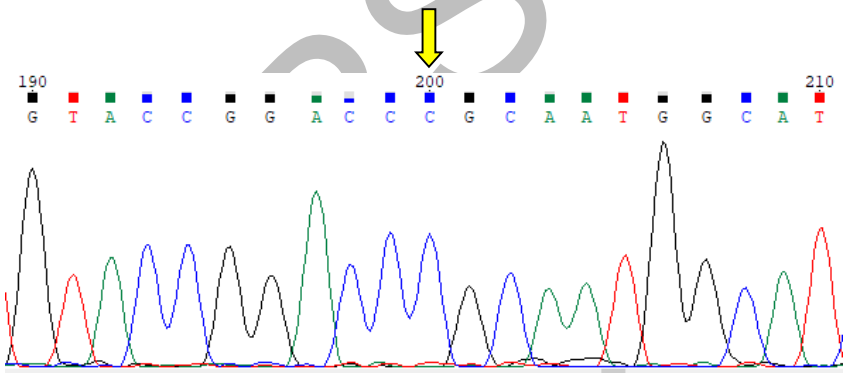


Fig. 3.12: Sequencing Chromatogram: Showing no mutation at chr5:149323933G>T position in exon 1 of *PDE6A* gene of proband of RP-121 Family.

3.7.6. Family RP-122:

Sanger`s sequencing of exon 1 of RP122P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. Moreover, neither any polymorphism nor other mutation was found in the sequence. The sequencing chromatogram is shown in fig 3.13.

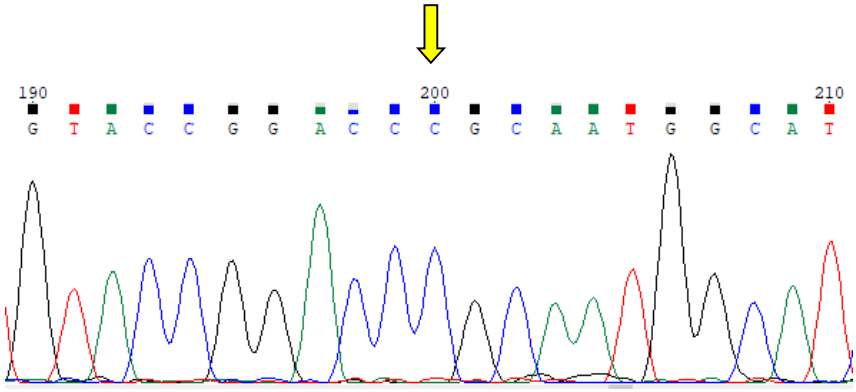


Fig. 3.13: Sequencing Chromatogram: Showing no mutation at chr5:149323933G>T position in exon 1 of *PDE6A* gene of proband of RP-122 Family.

3.7.7. Family RP-123:

Sanger`s sequencing of exon 1 of RP123P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. However, one polymorphism (c.331A>C) was found in the sequence. The sequencing chromatogram is shown in fig 3.14.

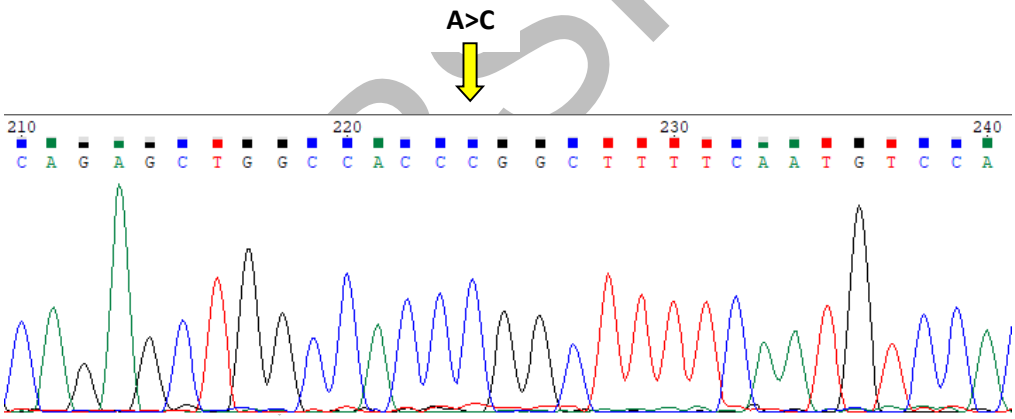


Fig. 3.14: Sequencing Chromatogram: Showing c.331A>C polymorphism (rs2277925) at chr5:149323906T>G position in exon 1 of *PDE6A* gene of proband of RP-123 Family.

3.7.8. Family RP-124:

Sanger`s sequencing of exon 1 of RP124P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. However, two mutations (c.274G>A and c.299G>A) were found in the sequence. The sequencing chromatogram is shown in fig 3.15.

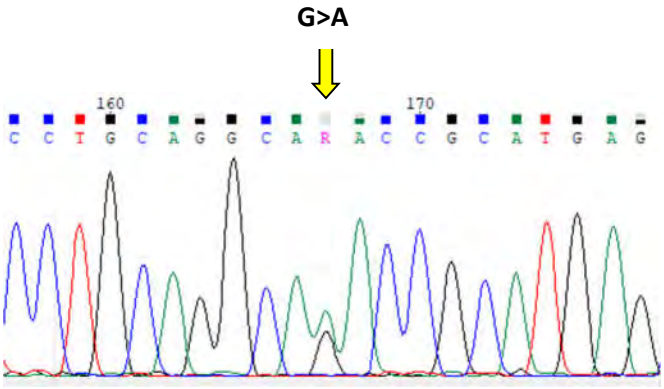


Fig. 3.15 (A): Sequencing Chromatogram: Showing c.274G>A mutation at chr5:149323963C>T position in exon 1 of *PDE6A* gene of proband of RP-124 Family.

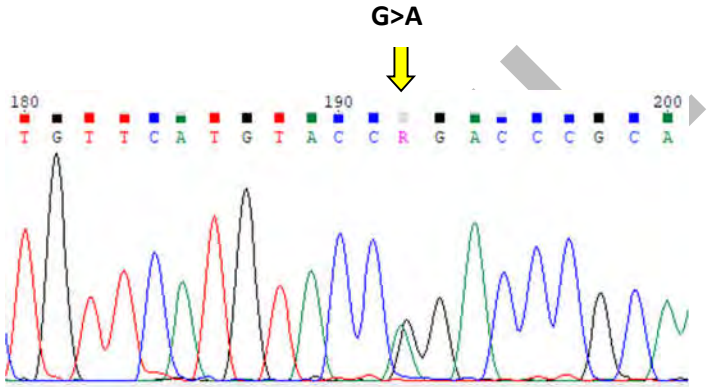


Fig. 3.15 (B): Sequencing Chromatogram: Showing c.299G>A mutation at chr5:149323938C>T position in exon 1 of *PDE6A* gene of proband of RP-124 Family.

3.7.9. Family RP-125:

Sanger`s sequencing of exon 1 of RP125P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. Moreover, neither any polymorphism nor other mutation was found in the sequence. The sequencing chromatogram is shown in fig 3.16.

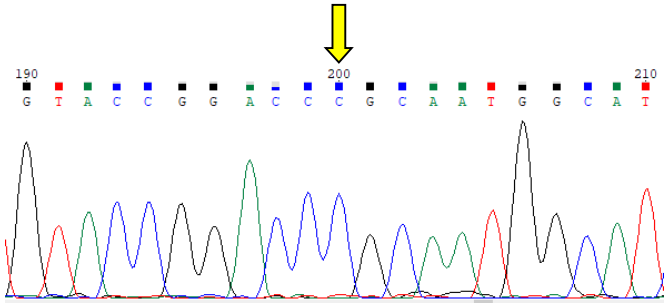


Fig. 3.16: Sequencing Chromatogram: Showing no mutation at chr5:149323933G>T position in exon 1 of *PDE6A* gene of proband of RP-125 Family.

3.7.10. Family RP-126:

Sanger`s sequencing of exon 1 of RP126P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. Moreover, neither any polymorphism nor other mutation was found in the sequence. The sequencing chromatogram is shown in fig 3.17.

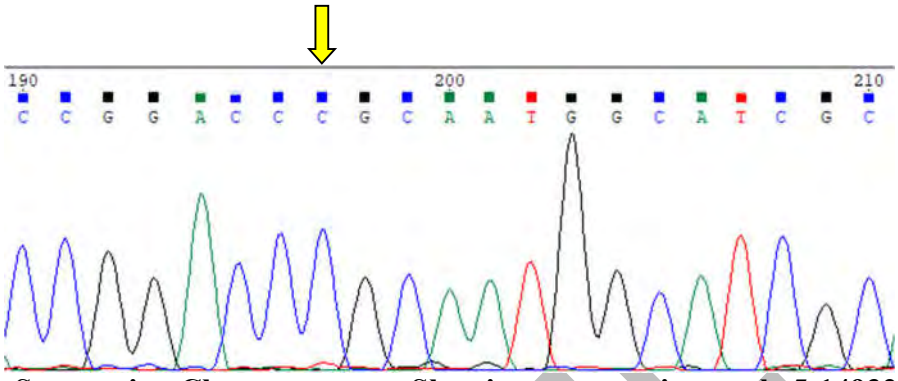


Fig. 3.17: Sequencing Chromatogram: Showing no mutation at chr5:149323933G>T position in exon 1 of *PDE6A* gene of proband of RP-126 Family.

3.7.11. Family RP-127:

Sanger`s sequencing of exon 1 of RP127P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. However, one polymorphism (c.331A>C) and two mutations (c.149A>C and c.150_151insA) were found in the sequence. The sequencing chromatogram is shown in fig 3.18.

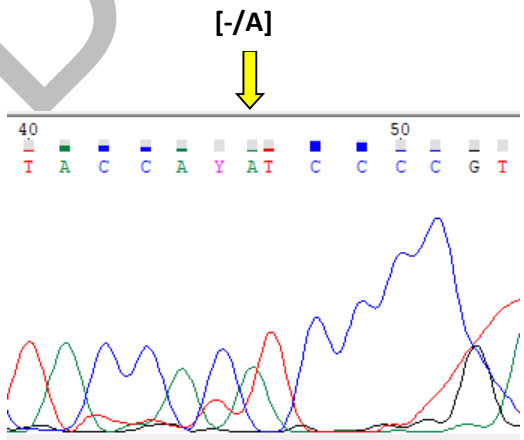


Fig. 3.18 (A): Sequencing Chromatogram: Showing c.150_151insA mutation at chr5:149324086insT position in exon 1 of *PDE6A* gene of proband of RP-127 Family.

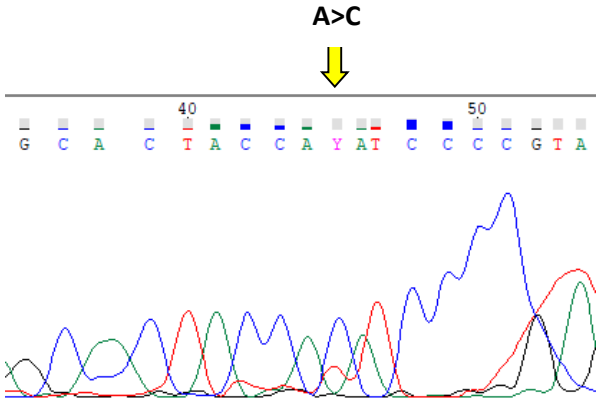


Fig. 3.18 (C): Sequencing Chromatogram: Showing c.149A>C mutation at chr5:149324088T>G position in exon 1 of *PDE6A* gene of proband of RP-127 Family.

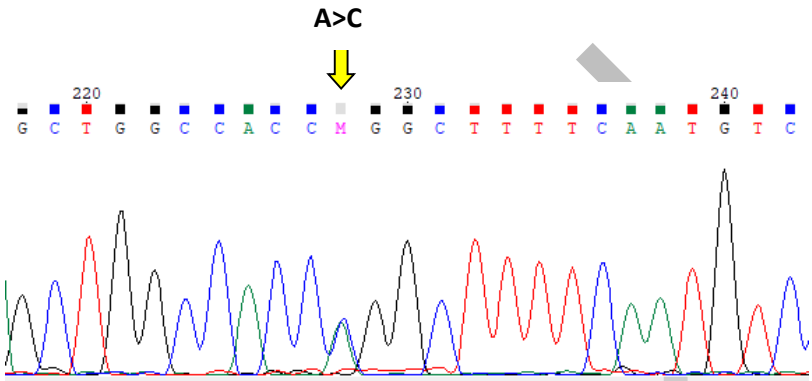


Fig. 3.18 (D): Sequencing Chromatogram: Showing c.331A>C polymorphism (rs2277925) at chr5:149323906T>G position in exon 1 of *PDE6A* gene of proband of RP-127 Family.

3.7.12. Family RP-128:

Sanger`s sequencing of exon 1 of RP128P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. Moreover, neither any polymorphism nor other mutation was found in the sequence. The sequencing chromatogram is shown in fig 3.19.

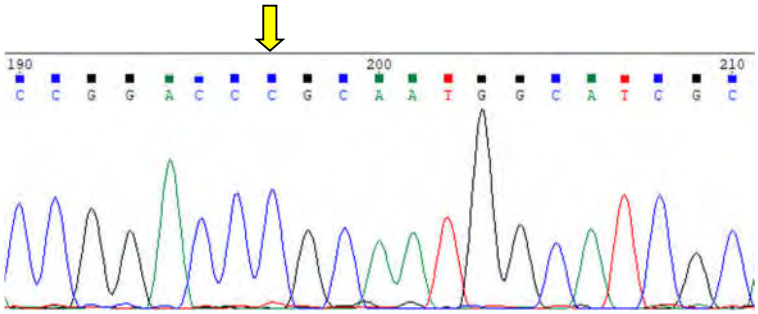


Fig. 3.19: Sequencing Chromatogram: Showing no mutation at chr5:149323933G>T position in exon 1 of *PDE6A* gene of proband of RP-128 Family.

3.7.13. Family RP-129:

Sanger`s sequencing of exon 1 of RP129P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. However, one polymorphism (c.331A>C) was found in the sequence. The sequencing chromatogram is shown in fig 3.20.

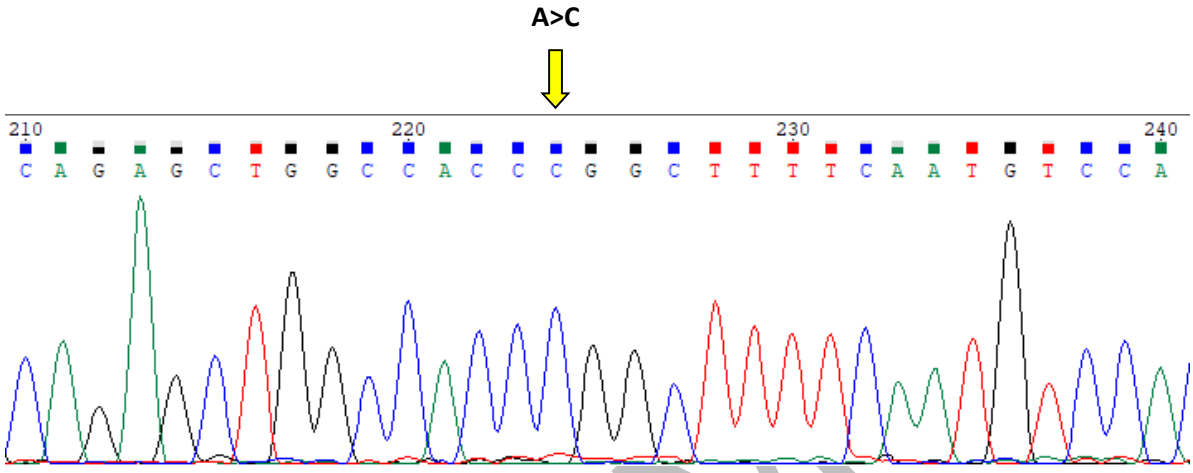


Fig. 3.20: Sequencing Chromatogram: Showing c.331A>C polymorphism (rs2277925) at chr5:149323906T>G position in exon 1 of *PDE6A* gene of proband of RP-129 Family.

3.7.14. Family RP-130:

Sanger`s sequencing of exon 1 of RP130P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. Moreover, neither any polymorphism nor other mutation was found in the sequence. The sequencing chromatogram is shown in fig 3.21.

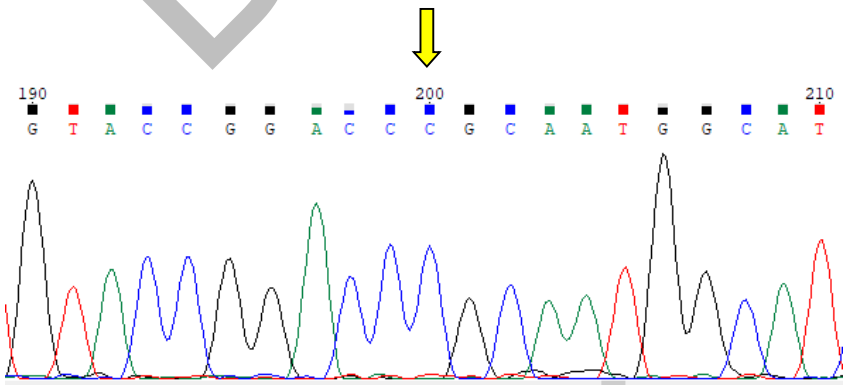


Fig. 3.21: Sequencing Chromatogram: Showing no mutation at chr5:149323933G>T position in exon 1 of *PDE6A* gene of proband of RP-130 Family.

3.7.15. Family RP-131:

Sanger`s sequencing of exon 1 of RP131P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. However, one polymorphism (c.331A>C) was found in the sequence. The sequencing chromatogram is shown in fig 3.22.

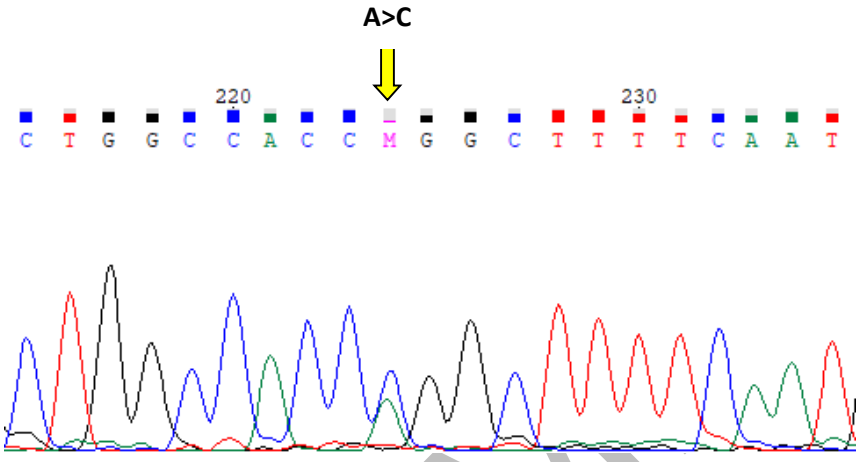


Fig. 3.22: Sequencing Chromatogram: Showing c.331A>C polymorphism (rs2277925) at chr5:149323906T>G position in exon 1 of *PDE6A* gene of proband of RP-131 Family.

3.7.16. Family RP-132:

Sanger`s sequencing of exon 1 of RP132P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. However, one polymorphism (c.331A>C) was found in the sequence. The sequencing chromatogram is shown in fig 3.23.

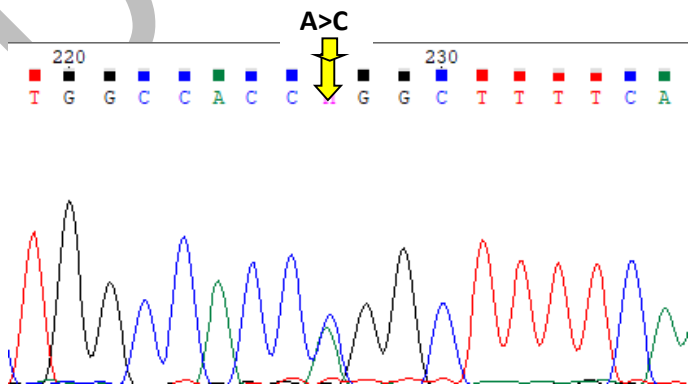


Fig. 3.23: Sequencing Chromatogram: Showing c.331A>C polymorphism (rs2277925) at chr5:149323906T>G position in exon 1 of *PDE6A* gene of proband of RP-132 Family.

3.7.17. Family RP-133:

Sanger`s sequencing of exon 1 of RP133P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. Moreover, neither any polymorphism nor other mutation was found in the sequence. The sequencing chromatogram is shown in fig 3.24.

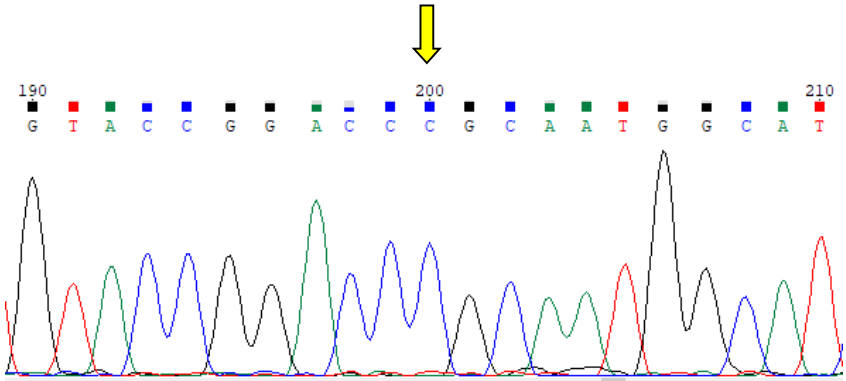


Fig. 3.24: Sequencing Chromatogram: Showing no mutation at chr5:149323933G>T position in exon 1 of *PDE6A* gene of proband of RP-133 Family.

3.7.18. Family RP-134:

Sanger`s sequencing of exon 1 of RP134P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. However, one polymorphism (c.331A>C) was found in the sequence. The sequencing chromatogram is shown in fig 3.25.

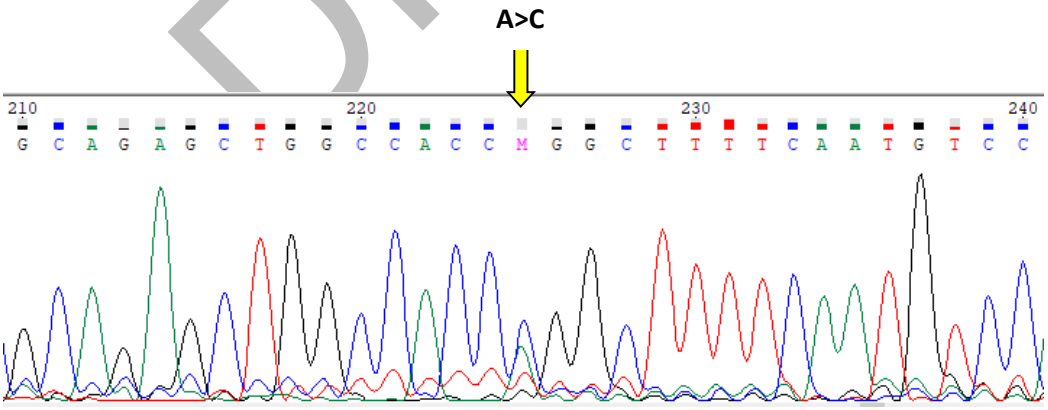


Fig. 3.25: Sequencing Chromatogram: Showing c.331A>C polymorphism (rs2277925) at chr5:149323906T>G position in exon 1 of *PDE6A* gene of proband of RP-134 Family.

3.7.19. Family RP-135:

Sanger`s sequencing of exon 1 of RP135P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. However, one polymorphism (c.331A>C) was found in the sequence. The sequencing chromatogram is shown in fig 3.26.

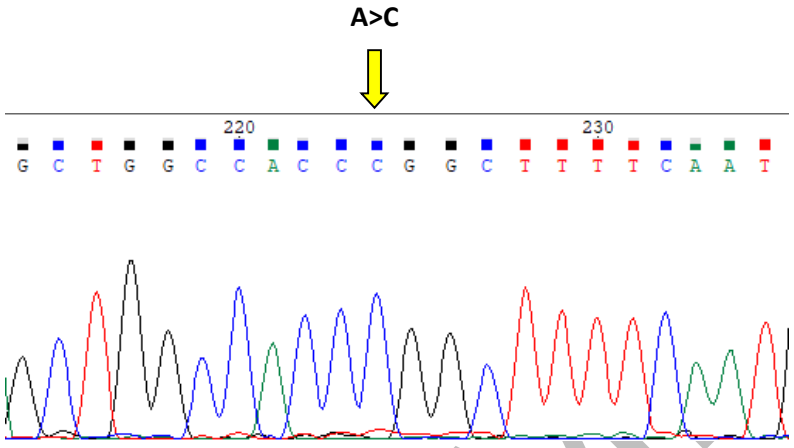


Fig. 3.26: Sequencing Chromatogram: Showing c.331A>C polymorphism (rs2277925) at chr5:149323906T>G position in exon 1 of *PDE6A* gene of proband of RP-135 Family.

3.7.20. Family RP-136:

Sanger`s sequencing of exon 1 of RP136P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. However, one polymorphism (c.331A>C) was found in the sequence. The sequencing chromatogram is shown in fig 3.27.

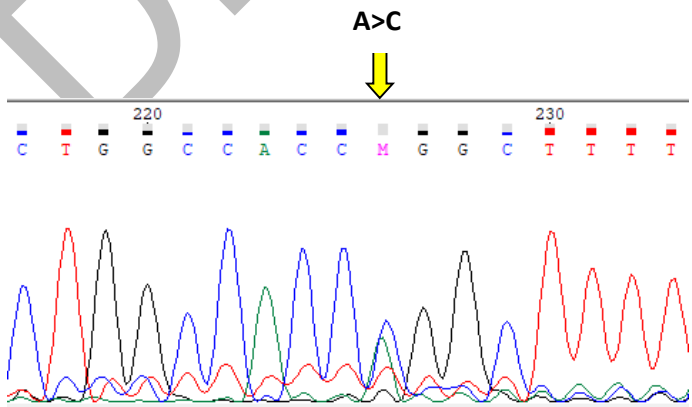


Fig. 3.27: Sequencing Chromatogram: Showing c.331A>C polymorphism (rs2277925) at chr5:149323906T>G position in exon 1 of *PDE6A* gene of proband of RP-136 Family.

3.8.10. Family RP-137:

Sanger`s sequencing of exon 1 of RP137P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. However, one polymorphism (c.331A>C) was found in the sequence. The sequencing chromatogram is shown in fig 3.28.

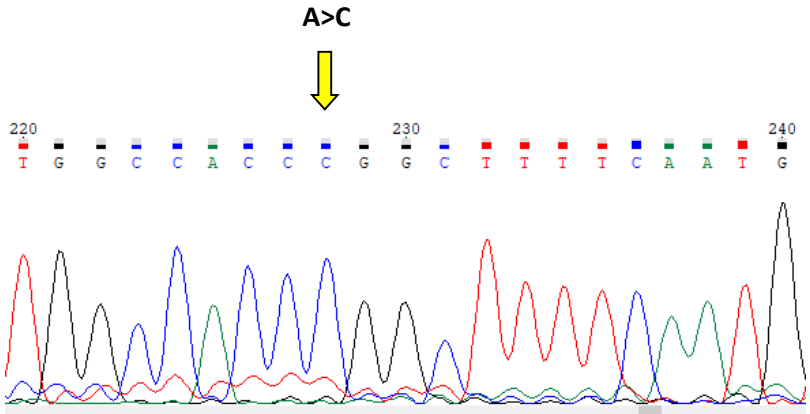


Fig. 3.28: Sequencing Chromatogram: Showing c.331A>C polymorphism (rs2277925) at chr5:149323906T>G position in exon 1 of *PDE6A* gene of proband of RP-137 Family.

3.8.11. Family RP-138:

Sanger`s sequencing of exon 1 of RP138P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. However, one polymorphism (c.331A>C) was found in the sequence. The sequencing chromatogram is shown in fig 3.29.

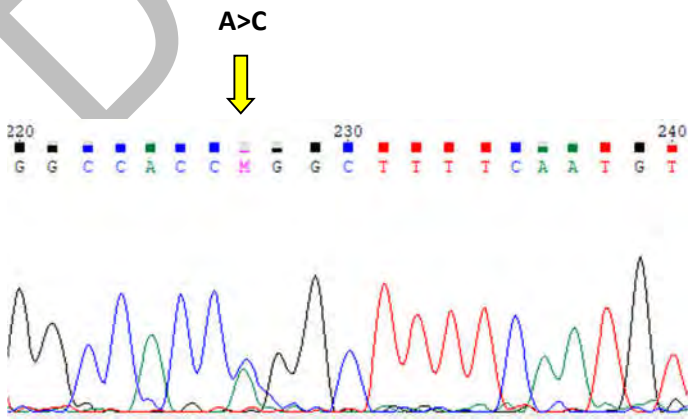


Fig. 3.29: Sequencing Chromatogram: Showing c.331A>C polymorphism (rs2277925) at chr5:149323906T>G position in exon 1 of *PDE6A* gene of proband of RP-138 Family.

3.8.12. Family RP-139:

Sanger`s sequencing of exon 1 of RP139P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. Moreover, neither any polymorphism nor other mutation was found in the sequence. The sequencing chromatogram is shown in fig 3.30.

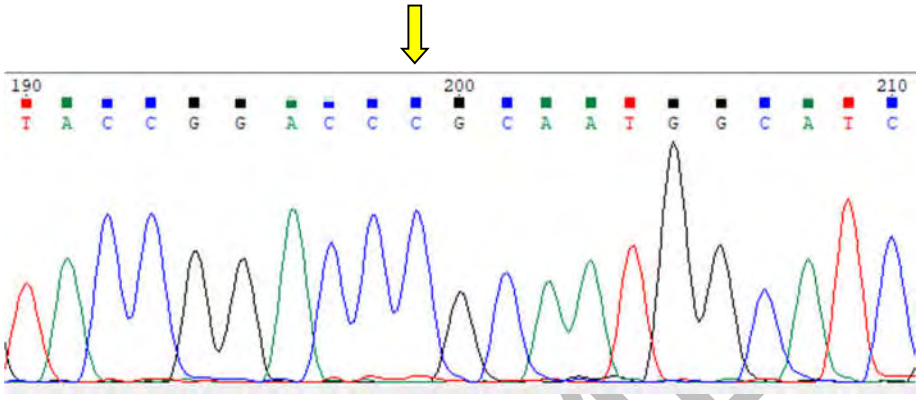


Fig. 3.30: Sequencing Chromatogram: Showing no mutation at chr5:149323933G>T position in exon 1 of *PDE6A* gene of proband of RP-139 Family.

3.8.13. Family RP-140:

Sanger`s sequencing of exon 1 of RP140P1 proband shows that c.304C>A, p.R102S mutation was not found in sequence. Moreover, neither any polymorphism nor other mutation was found in the sequence. The sequencing chromatogram is shown in fig 3.31.

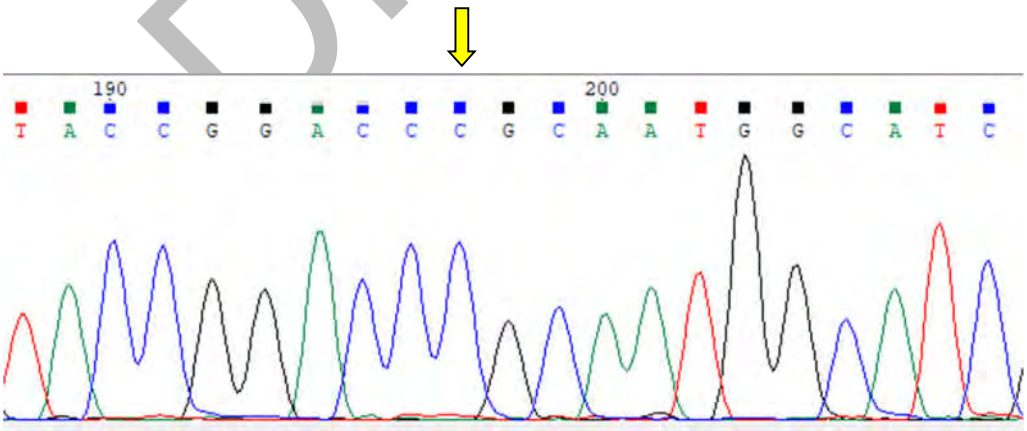


Fig. 3.31: Sequencing Chromatogram: Showing no mutation at chr5:149323933G>T position in exon 1 of *PDE6A* gene of proband of RP-140 Family.

Table 3.3: Identified Polymorphism and Mutations in Enrolled RP Families

Exon No.	Genomic Location	Physical Location	AA Change	rs. ID	Zygosity	Mutation Taster	Status
1	c.331A>C	chr5:149323906T>G	No AA changes	rs2277925	Heterozygous	Polymorphism	Reported
1	c.274G>A	chr5:149323963C>T	D92N	rs199924410	Heterozygous	Mutation	Reported
1	c.299G>A	chr5:149323938C>T	R100Q	rs199738915	Heterozygous	Mutation	Reported
1	c.386A>G	chr5:149323851T>C	D129G	N/A	Heterozygous	Mutation	Novel
1	c.150_151insA	chr5:149324086insT	S51I	N/A	Homozygous	Mutation	Novel
1	c.149 A>C	chr5:149324088T>G	H50P	N/A	Homozygous	Mutation	Novel

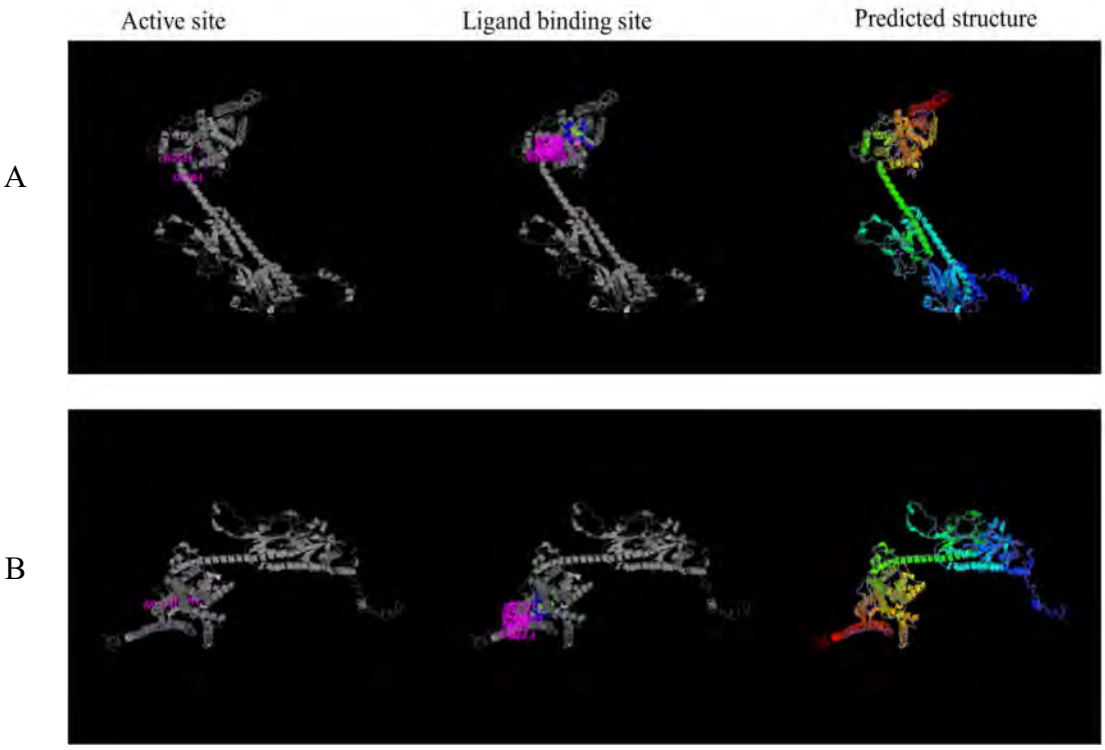


Fig. 3.4: Structure of *PDE6A* protein (A) Wild and (B) Mutated generated by I-TASSER.

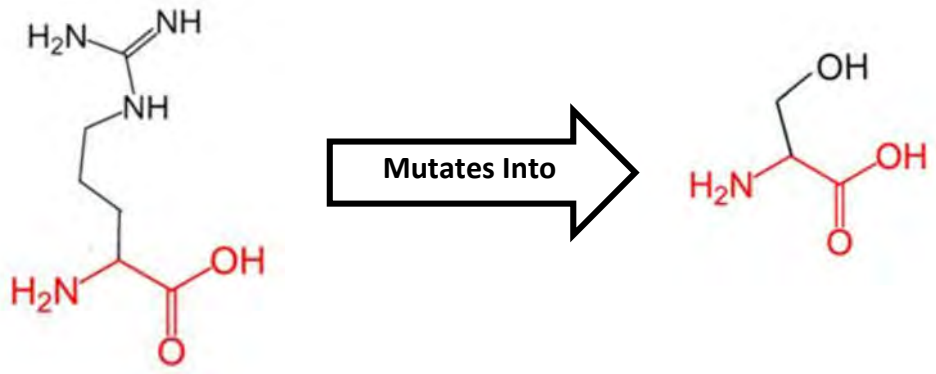


Fig. 3.5: Structure of wild and substituted residue at position 102 of *PDE6A* protein.

Chapter No. 4:

DISCUSSION

Retinitis pigmentosa (RP) is a genetic disorder characterized by the progressive degeneration of retina of eyes mainly the photoreceptor layer. It is a heterogenous group of retinal diseases which involves inherited neurodegenerative diseases that effects both structures and functions of RPE and photoreceptors (Newton *et al.*, 2020). Approximately more than 2.5 million peoples are affected from RP across the globe and its prevalence is about 1:4000 worldwide (Coussa *et al.*, 2019; Daggula *et al.*, 2020). There is a gradual apoptotic cell loss which leads to the reduction in the retinal functions and ultimately cause retinal atrophy and blindness. Visual impairment appears in initial stages with certain degree of gradual night blindness (NB) and loss of visual field and ultimately cause retinal atrophy and blindness (Sudharsan *et al.*, 2019).

Eye examination have revealed, weakening of retinal vessels, abnormal fundus with bone spicules accumulation, waxy pallor of optic disc, macular degeneration, and arterial attenuation (Nakazawa *et al.*, 2019). The severity of visual impairment and other disorders depends upon the degree of disfunction and degeneration of photoreceptor cells in retina (Meng *et al.*, 2020). The age of onset of RP, varies and depends upon its type. Mostly it ranges from the early childhood to the late adulthood (Kim *et al.*, 2021; Murakami *et al.*, 2020). The severity of RP also varies from mild unnoticeable loss of visual field to tunnel vision and impaired central vision. The most common pattern of RP is that it is steady and slowly deterioration of retina or RPE (Gagliardi *et al.*, 2019).

The inheritance mode of RP can be categorized as Autosomal dominant RP (adRP), Autosomal recessive RP (arRP) and X-Linked RP. Autosomal recessive RP (arRP) is the most frequently reported form of RP (Cehajic-Kapetanovic *et al.*, 2020; Kuehlewein *et al.*, 2021). It is frequently reported in Saudi, South Indian, Israeli and Pakistani population with high rate of endogamy and consanguinity (Dan *et al.*, 2020). Globally, among all the reported cases, 30% cases are of adRP, 20% are of arRP, 15% are of X-linked RP, 5% are of recessive LCA and the remaining 30% are of isolated RP (O'Neal *et al.*, 2022; Zeviani *et al.*, 2021). The isolated cases

have mostly autosomal recessive mutations but de novo mutations. Generally, X Linked form of RP is more severe as compared to autosomal recessive RP (Dan *et al.*, 2020).

In past decades, a rapid increase in RP cases has been reported. U.S have been reported about 6500 non-syndromic RP cases in last 3 years (Cross *et al.*, 2022). In 2010, the number of RP cases reported in South Asia are, 15,482 cases in Afghanistan, 58,963 cases in Bangladesh, 1603 cases in Bhutan, 391,570 cases in India, 9318 cases in Sri Lanka and 58,528 cases in Pakistan (Bouzidi *et al.*, 2021; Sun *et al.*, 2021; Thapa *et al.*, 2020). In 2020, an increase in these numbers have been reported such that, 21,821 cases in Afghanistan, 83,293 cases in Bangladesh, 3887 cases in Bhutan, 588,320 cases in India, 13,931 cases in Sri Lanka and 92,762 cases in Pakistan. It is reported that, autosomal recessive RP is the most common form of retinal dystrophies in Pakistani population (Moore *et al.*, 2020). Studies on Pakistani population shows that 64% cases are of autosomal recessive retinitis pigmentosa, 18% are of autosomal recessive Leber Congenital Amerosis, 8% are of autosomal recessive congenital stationary night blindness and 10% are of autosomal recessive cone-rod dystrophy (Maria *et al.*, 2015; Shahzad *et al.*, 2013).

Consanguinities are found common in many countries, which play a significant role in the inheritance of autosomal recessive disorders (Al-Bdour *et al.*, 2020). Some of the common disorders that are found in off-springs of consanguineous marriages are Thalassemia, Cystic fibrosis, Down's syndrome and Visual impairments (Oniya *et al.*, 2019). It is studied that the ratio of genetic disorders is twice in the children due to cousin marriages as compared to children of non-related parents (Youssefian *et al.*, 2019). It is reported that in Pakistan, the frequency of cousin marriages is about 80% that contributes 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). Pakistani population is genetically less investigated for inherited disorders due to their low economic status (Awan *et al.*, 2020).

In present study twenty-four consanguineous families were enrolled having different origin i.e. KPK, Punjab, Kashmir and Gilgit Baltistan. Each family had multiple affected individuals. The diagnosis of RP in these families was confirmed by ophthalmologists at Al-Shifa Eye Trust Hospital, Rawalpindi, Pakistan. All enrolled twenty-four families showed

autosomal recessive pattern of inheritance of RP phenotype. The ages of enrolled affected individual ranged from 5 to 70 years. In this study the hotspot exon i.e. exon 1 of *PDE6A* having location for c.304C>A, p.R102S mutation was selected, based on previous reports from Pakistan (Hayashi *et al.*, 2021; Khan *et al.*, 2021; Kuehlewein *et al.*, 2022). No c.304C>A, p.R102S missense mutation was found in sequenced probands of all enrolled families. This missense variant results in improper folding of protein as reported previously (Li *et al.*, 2022). However, one previously reported polymorphism i.e. c.331A>C (heterozygous) in RP120, RP123, RP127, RP129, RP131, RP132, RP134, RP135, RP136, RP137, RP138, two previously reported mutations i.e. c.274G>A, c.299G>A (heterozygous) in RP124, one novel mutation i.e. c.386A>G (heterozygous) in RP117 and two novel mutations i.e. c.150_151insA, c.149A>C (homozygous) in RP127 were found in this study.

The age of onset in all enrolled families was in first decade of life. Fundus examination of all enrolled patients revealed attenuated vessels, waxy pallor, peripheral bony spicules deposit and short sightedness, which are typical features of RP (Verbakel *et al.*, 2018). All the affected individuals experienced progressive night blindness as reported previously for RP cases. Among 24 families, 11 were found to be syndromic RP families while 13 were found to be non-syndromic RP families. Many of the signs, symptoms and fundus appearance are found similar to those describe in previously reported cases (Khan *et al.*, 2021; Nwosu *et al.*, 2020; Schön *et al.*, 2017).

In syndromic RP the autosomal recessive pattern of inheritance accounts for about 50-65% while in case of non-syndromic RP, it accounts for about 55-60% (Bruninx *et al.*, 2020). In arRP more than 60 genes are involve, among these genes *PDE6A* gene has significant contributions in arRP genetic load (Cross *et al.*, 2022). *PDE6A* gene is located on chromosome 5q32. It consists of total 22 exons that encodes for 860 amino acids. The *PDE6A* gene encodes for alpha subunit of cGMP –phosphodiesterase, an enzyme found in rod photoreceptors (Chen *et al.*, 2018). The *PDE6A* gene has been linked to about 50 pathogenic variants the majority of which are single base substitution (60%), resulting in nonsense or missense variants (Bujakowska *et al.*, 2020). In Pakistani population the contribution of *PDE6A* mutation in autosomal recessive RP is 2% to 3% (Ali *et al.*, 2022; Iqbal *et al.*, 2019).

The variant c.304C>A, p.Arg102Ser was reported for the first time in Danish population (Dawood *et al.*, 2021). In a study on 350 Pakistani consanguineous families by found 3 RP affected families carrying c.304C>A (Ullah *et al.*, 2016). This variant was also reported by (Dawood *et al.*, 2021; Khan *et al.*, 2021; Zafar *et al.*, 2017) in Pakistani population. The arginine at position 102 is extremely conserved in many species and is found in cGMP specific Phospho-diesterases, adenylyl cyclases and FhlA (GAF) domain of *PDE6A* protein. The three other amino acid substitutions reported at codon 102 are serine (p.Arg102Ser), cysteine (p.Arg102Cys) and histidine (p.Arg102His). All these substitutions are pathogenic and interrupts the GAF1 domain function, changes protein functions and leads to retinal anomalies (Khan *et al*., 2021).

The in-silico analysis revealed that the c.304C>A, p.Arg102Ser mutant residue was different from wild type in charge, size, and hydrophobicity. The size of substituting residue was smaller than that of wild type while the charge was neutral unlike positively charge wild type reside. Furthermore, the mutant residue was more hydrophobic than that of wild type residue. Change in size and charge results in interaction loss with other residues or molecules. Increase in hydrophobicity leads to improper folding of protein due to hydrogen bonds loss as shown in fig 3.18.

c.331A>C polymorphism (rs2277925) in exon 1 of *PDE6A* gene, found in this study was previously reported by (Riazuddin *et al.*, 2006) in four RP families (61019, 61021, 61074, and 61081) in a study on Pakistani population. This polymorphism leads to no change in amino acids sequence i.e. Arg111Arg. The fundus of patients have boney spicule pigmentation, attenuated arteries, waxy pallor of optic disc and non-detectable ERG readings (Riazuddin *et al.*, 2006). It is also reported that this polymorphism is found in heterozygous conditions in all the families included in studies on Pakistani population (Ali *et al.*, 2011; Riazuddin *et al.*, 2006).

c.150_151insA, pS51I mutation in exon 1 of *PDE6A* gene, found in this study was not reported previously in any population. This novel mutation was found in RP127 family. According to mutation taster (https://www.mutationtaster.org/), this mutation leads to the change of Serine at 51st position with Isoleucine (Ser51Ile). According to the I-Mutant v2.0 (https://folding.biofold.org/cgi-bin/i-mutant2.0.cgi), this change will lead to increase the stability of mutant protein.

c.149A>C, p.H50P mutation in exon 1 of *PDE6A* gene, found in this study was not reported previously in any population. This novel mutation was found in RP127 family. According to mutation taster (https://www.mutationtaster.org/), this mutation leads to the change of Histidine at 50th position with Proline (His50Pro). According to the I-Mutant v2.0 (https://folding.biofold.org/cgi-bin/i-mutant2.0.cgi), this change will lead to increase the stability of mutant protein.

c.386A>G, p.D129G mutation in exon 1 of *PDE6A* gene, found in this study was not reported previously in any population. This novel mutation was found in RP117 family. According to mutation taster (https://www.mutationtaster.org/), this mutation leads to the change of Aspartic acid at 129th position with Glycine (Asp129Gly). According to the I-Mutant v2.0 (https://folding.biofold.org/cgi-bin/i-mutant2.0.cgi), this change will lead to decrease the stability of mutant protein.

c.274G>A, p.D92N mutation (rs199924410) in exon 1 of *PDE6A* gene, found in this study was previously reported by (Karali *et al.*, 2019) in two RP families (RP10 and RP24) in a study on Pakistani population. This reported mutation was found in RP124 family. According to mutation taster (https://www.mutationtaster.org/), this mutation leads to the change of Aspartic acid at 92nd position with Asparagine (Asp92Asn). According to the I-Mutant v2.0 (https://folding.biofold.org/cgi-bin/i-mutant2.0.cgi), this change will lead to decrease the stability of mutant protein. The fundus of patients have boney spicule pigmentation, attenuated arteries, waxy pallor of optic disc and non-detectable ERG readings (Babar *et al.*, 2022). It is also reported that this mutation was found in heterozygous conditions in all the families included in studies on Pakistani population (Jaffal *et al.*, 2021).

c.299G>A, p.R100Q mutation (rs199738915) in exon 1 of *PDE6A* gene, found in this study was previously reported by (Watson, 2022) in four RP families (RP56, RP61, RP63 and RP67) in a study on Pakistani population. This reported mutation was found in RP124 family. According to mutation taster (https://www.mutationtaster.org/), this mutation leads to the change of Arginine at 100th position with Glutamine (Arg100Gln). According to the I-Mutant v2.0 (https://folding.biofold.org/cgi-bin/i-mutant2.0.cgi), this change will lead to decrease the stability of mutant protein. The fundus of patients have boney spicule pigmentation, attenuated arteries,

waxy pallor of optic disc and non-detectable ERG readings (Ali *et al.*, 2008). It is also reported that this mutation was found in heterozygous conditions in all four the families included in studies on Pakistani population (Burhan *et al.*, 2021).

The result of this study reveals that c.304C>A, p.R102S mutation variant is may not be a common mutation in Pakistani population as it was not detected in 24 families. However, one previously reported polymorphism i.e. c.331A>C in heterozygous condition in RP120, RP123, RP127, RP129, RP131, RP132, RP134, RP135, RP136, RP137 and RP138 families, two previously reported mutations i.e. c.274G>A, c.299G>A in heterozygous condition in RP124, one novel mutation i.e. c.386A>G in heterozygous condition in RP117 and two novel mutations i.e. c.150_151insA, c.149A>C in homozygous condition in RP127 family were found in this study. All RP families should be initially screened for other variants of *PDE6A* gene. Genetic counseling was given to all the enrolled and highly inbred families to discourage the cousin marriages in RP affected families to limit the incidences of disease in the future generations. The findings of this study, necessitates a public awareness effort regarding inherited recessive disorders prevailing in Pakistani population to lessen the burden of disease as well as mortality and morbidity linked to such disease in future. The study of recessive genetic disorders in our community might yield new insights into mechanism of disease and aid in the development of therapeutic regimes.

CONCLUSION

It is concluded that incidence of recessive forms of Retinitis Pigmentosa is high in our study population. Among multiple reported RP genes, *PDE6A* gene is highly associated with different forms of RP and p.Arg102Ser is a common mutation of this gene but we could not identify that in our study. The result of this study revealed one previously reported polymorphism i.e. c.331A>C in heterozygous condition, two previously reported mutations i.e. c.274G>A, c.299G>A in heterozygous condition, one novel mutation i.e. c.386A>G in heterozygous condition and two novel mutations i.e. c.150_151insA and c.149A>C in homozygous condition in exon 1 of *PDE6A* gene. All RP families should be initially screened for other variants of *PDE6A* gene. Genetic counseling should be given to all the affected families and cousin marriages should be highly discouraged in RP affected families and to limit the incidences of disease in the future generations.

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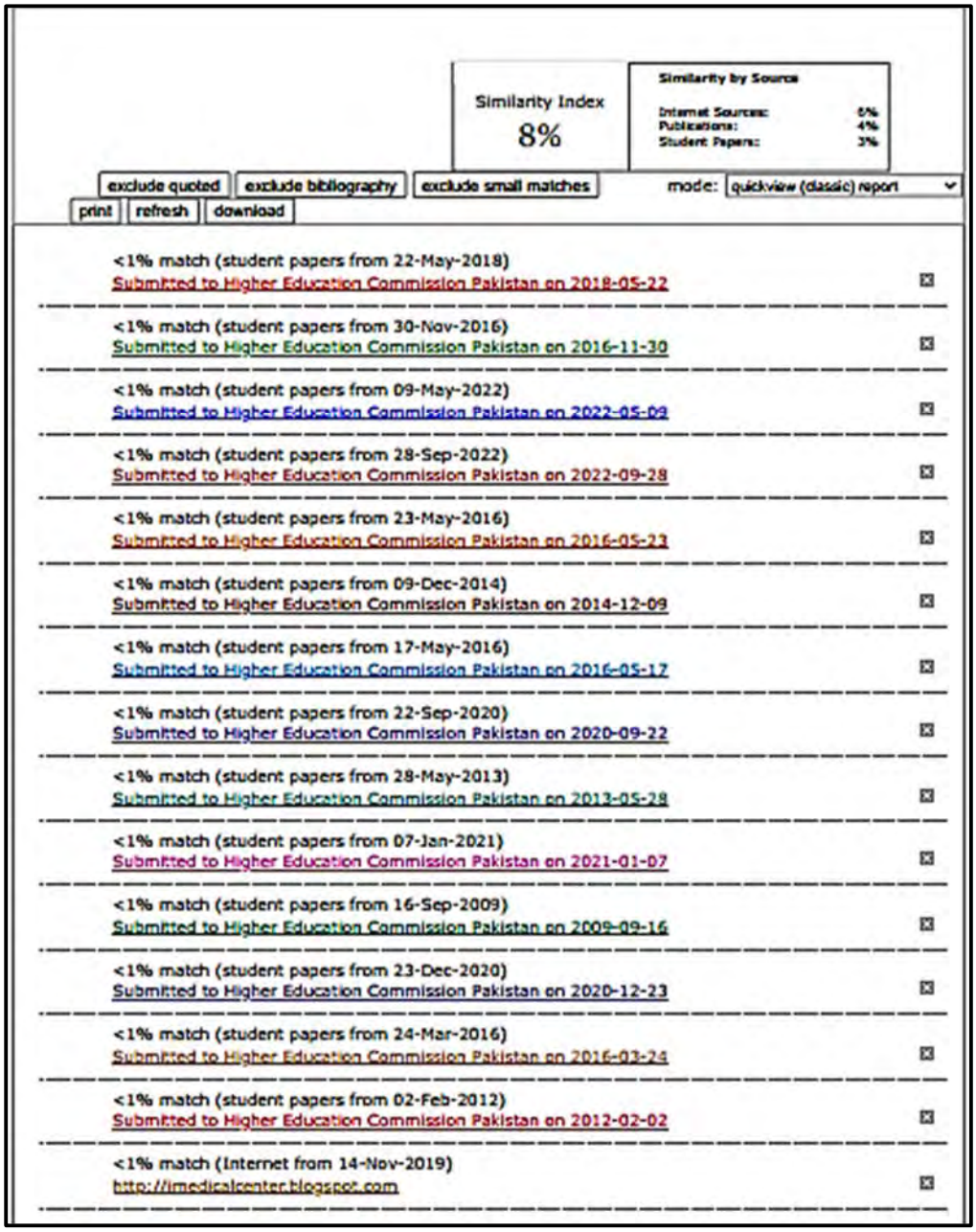

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