Investigating the Molecular Basis of Bardet-Biedl Syndrome in Familial Cases

By

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M. Phil Molecular Biology

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Department of Zoology

Faculty of Biological Sciences

Quaid-i-Azam University Islamabad

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

In

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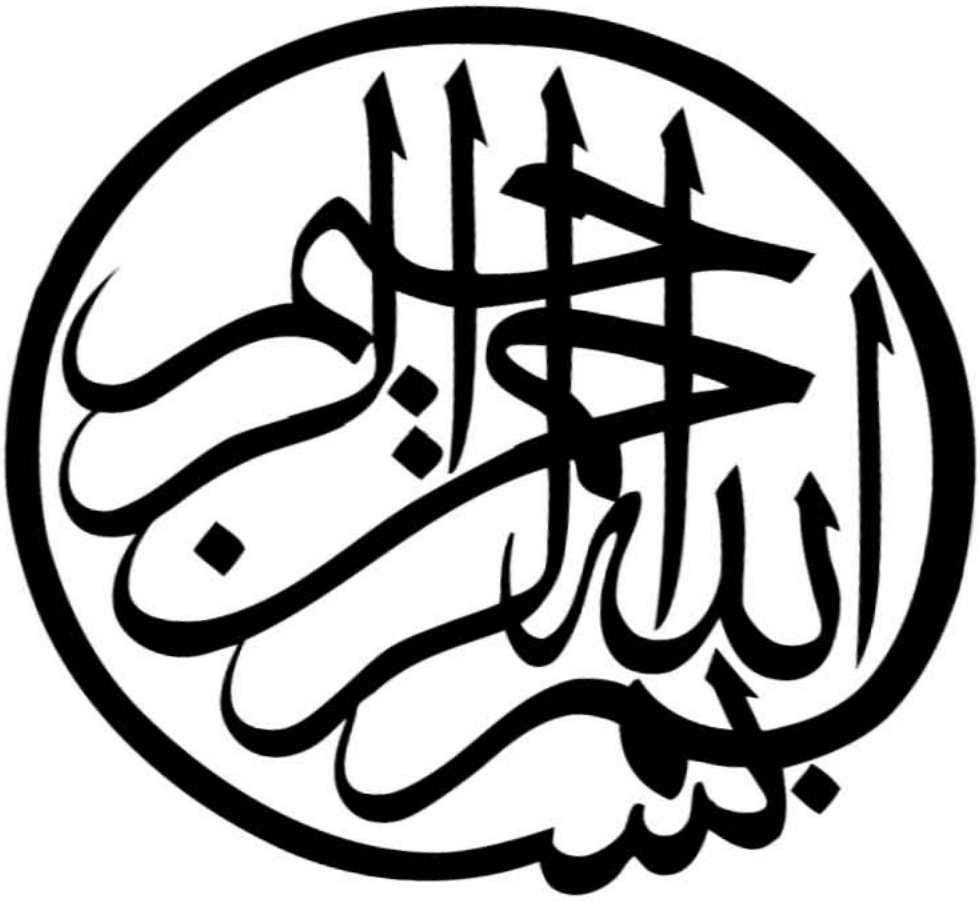
بسم الله الرحمن الرحيم

CERTIFICATE

This dissertation "Investigating the Molecular basis of Bardet-Biedl Syndrome in familial cases" submitted by **Mr. Muhammad Shoaib**, is accepted in its present form by the Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad as satisfying the thesis requirement for the degree of Master of Philosophy in Molecular Biology.

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DECLARATION

I **Muhammad Shoaib**, student of **M. Phil. Molecular Biology**, Session **2021-2023**, hereby declare that the material and information contained in this thesis titled "**Investigating the Molecular basis of Bardet-Biedl Syndrome in familial cases"** 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.

_____

Muhammad Shoaib

DEDICATION

This study is heartedly dedicated to My Beloved Parents, Family and whole

Kashmiri Nation

ACKNOWLEDGEGEMENT

In the name of **Almighty Allah**, the most Gracious and Most Merciful. 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. I experienced so much during this process, not only from the academic aspects but also from the aspect of personality. My humble gratitude to the **Holy Prophet Muhammad (S.A.W)** whose way of life has been continuous guidance for me.

First and foremost, I would like to sincerely thank my supervisor **Dr. Sabika Firasit**, Associate Professor of Zoology, Quaid-i-Azam University Islamabad, for her guidance, understanding, patience and most importantly she has provided positive encouragement and warm spirit to complete this thesis. It has been a great pleasure and honor to have her as my supervisor.

I also thank to my **Parents** for their continuous support and their pray help me to reach this destination. And I express my thanks to my **Siblings**, as they all encouraged me during my MPhil studies. I also thank to **Quaidian's Especially Kashmiri Quaidian's** for their moral support.

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Muhammad Shoaib

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ABBREVATIONS OF TERMS AND ACRONYMS

BBS	Bardet-Biedl Syndrome
PD	Polydactyly
RCD	Rod/Cone Dystrophy
RP	Retinitis Pigmentosa
ERG	Electroretinography
OS	Outer Segment
IFT	Intraflagellar Transport
IS	Inner Segment
Shh	Sonic Hedgehog
PCP	Planar Cell Polarity
mTOR	Mammalian Target of Rapamycin
LCA	Leber Congenital Amaurosis
RD	Retinal Detachment
GPCR	G-protein Coupled Receptors
PC	Polycystin
LOF	Loss-of-Function
ARF	ADP-ribosylation Factor
ACY1	Aminoacylase-1
CFAP	Cilia and Flagella Associated Protein
CEP	Centrosomal Protein
SCLT1	Sodium Channel and Clathrin Linker 1
SCAPER	S-phase cyclin A associated protein in ER
LOF	Loss-of-Function

CNV	Copy Number Variants
SNP	Single Nucleotide Polymorphism
NGS	Next Generation Sequencing
LCA	Leber Congenital Amaurosis
RBC	Red Blood Cells
PCR	Polymerase Chain Reaction

ABSTRACT

Bardet-Biedl (BBS) syndrome is a ciliopathy and it was first time identified by Laurence and Moon in 1866. BBS syndrome is recognized by its primary and secondary features. Rod/cone dystrophy (RCD), polydactyly (PD), obesity, genetal defects, renal abnormalities and learning problems are the primary symptoms of BBS. Developmental delay, dental issues, heart defects, speech problem, syndactyly or brachydactyly, poor coordination, olfactory defects, diabetes mellitus, hepertension, liver defects and craniofacial dismorphism are common BBS secondary symptoms. The appearance of symptoms of BBS are related to age, in early age only few symptoms are apparent other symptoms evolve during or after first decade of life, most individuals having polydactyly at birth appears healthy but at later stages of life they are diagnosed with BBS. BBS is thought to affect 1 in 150000 people worldwide, but its prevalence varies in isolated, inbred, and consanguineous cultures. Since around 26 genes are thought to be involved in BBS, many investigations have found that BBS patients experience a range of symptoms. Autosomal recessive mode of inheritance was observed in BBS syndrome by initial gene discovery studies but further more research complicated the genetics of BBS and prevailed incomplete penetrance and triallelic inheritance. *BBS10* and *BBS3/ARL6* are most mutated genes in Pakistan and India. The aim of present studys was to investigate the molecular basis of Bardet-Biedl syndrome in familial cases.Ethical approval was obtained from 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 Retinitus Pigmentosa (RP) by ophthalmologist and then after conducting detailed interview BBS families were selected. Partiticipating members were interviewed for family history of disease, pedigree drawing, and clinical records were collected from affected and unaffected members after written consent. The genomic DNA was extracted for genetic analysis. Primers were designed to amplify exon 2 and 5 of *ARL6* gene for mutation analysis. After the amplification of selected exon, the amplified products were purified and sent for Sanger's sequencing. Sequencing data identified previously reported mutation c.281T>C causing (p.I94T) in exon 5 of *ARL6* gene in enrolled family RP 43. However, other previously reported and novel variants were identified in exon 5 of *ARL6* gene but no variation was observed in exon 2 of *ARL6* gene of selected families. For families in which no disease causing variant was identified, screening of remaining exon of *ARL6* and other genes should be performed to identify molecular genetic defect. Results of this study also showed that consanguinity contributes to high incidence of recessively inherited disorders including BBS in our population. For this reason genetic counseling was provided to all participating families.

Chapter No. 1:

INTRODUCTION

1.7. Bardet-Biedl Syndrome (BBS)

Bardet-Biedl syndrome is a ciliopathy that is mostly recognized by its defining traits such as polydactyly (PD), renal defects, rod/cone dystrophy (RCD), hypogonadism and obesity. Different BBS gene mutations results in varying disease symptoms (P. Beales, Elcioglu, Woolf, Parker, & Flinter, 1999).

1.1.1 History of BBS Syndrome

In 1866 a family consisting of four siblings having Retinitis Pigmentosa (RP), intellectual disability and obesity and later on development of spastic paraparesis was fist time reported by Laurence and Moon. Then in 1920 and 1922, Bardet and Biedel separately reported two families with RP, obesity and polydactyly. From 1925 this syndrome was known as Laurence-Moon-Bardet-Biedel syndrome but there was a contradiction weather they were same entity or different, later on these were confirmed as two entities. Mutations in BBS genes are responsible for both of these syndromes and now a day these are commonly known as BBS (Bardet, 1995; P. Beales *et al*., 1999; Biedl, 1995; Forsythe, Kenny, Bacchelli, & Beales, 2018; Laurence, 1866; Moore *et al*., 2005).

1.1.2 Epidemiology

A rare hereditary syndrome called Bardet-Biedl syndrome (BBS) leads to severe multiple organ dysfunction. BBS is thought to affect 1 in 150000 people worldwide, but its prevalence varies in isolated, inbred, and consanguineous cultures (Forsythe & Beales, 2013).

Its occurrence is different in different communities some isolated communities has high occurrence as in North America and Europe its occurrence is below 1:100000 and in Newfoundland its occurrence is 1:18000 (Forsythe & Beales, 2013; Moore *et al*., 2005; Sheffield, 2004). BBS occurrence in Middle East is high almost 1:13500 in Bedouin communities and in some other families of other populations as in Faroe Island its prevalence is 13 patients in 48000 population almost 1:3700 frequency in island (Farag & Teebi, 1989; T Duelund Hjortshøj, Grønskov, Brøndum-Nielsen, & Rosenberg, 2009; M'hamdi, Ouertani, Maazoul, & Chaabouni-Bouhamed, 2011). Jews are most genetically studied population but until now not subjected to BBS epidemiological research (Fedick *et al*., 2014). Lower number of BBS cases is reported in Eastern Europe, Africa, Asia and South America and there is need of systematic BBS studies in these regions (Hirano *et al*., 2015; Saadullah Khan *et al*., 2013; Solmaz *et al*., 2015; Suspitsin *et al*., 2015; Xing *et al*., 2014). The prevalence frequency of BBS in Pakistan is still not reported.

1.1.3 Diagnosis of BBS

PD and renal defects are illustrated by the antenatal imaging but BBS diagnosis is not possible until the patient starts to attain the visual problem especially RCD. The diagnosis of BBS is mainly dependent on seminial study of Beals *et al*. [1999] and mainly according to phenotype (Forsythe & Beales, 2013). Multiple varying disease symptoms are reported in articles observed among different patients so the diagnosis depends on primary and secondary symptoms (P. Beales *et al*., 1999; Forsythe & Beales, 2013; M'hamdi, Ouertani, & Chaabouni-Bouhamed, 2014).The appearance of symptoms of BBS are related to age, in early age only few symptoms are apparent other symptoms evolve during or after first decade of life, most individuals having polydactyly at birth appears healthy but at later stages of life they are diagnosed with BBS. RCD was reported in 93% BBS patients in a study but patients below 8 were not having RCD (P. Beales *et al*., 1999).

1.1.4 Pattern of inheritance

Autosomal recessive mode of inheritance was observed in BBS syndrome by initial gene discovery studies but further more research complicated the genetics of BBS and prevailed incomplete penetrance and triallelic inheritance. Studies have shown individuals with biallelic gene mutation carriers for BBS that were healthy at investigation suggesting incomplete penetrance. Studies also showed BBS patients with homozygous mutation in one gene and heterozygous mutation in other gene for BBS suggesting triallelic inheritance (P. L. Beales *et al*., 2003; Estrada-Cuzcano *et al*., 2012; Katsanis *et al*., 2001; Kwitek-Black *et al*., 1993; Leppert *et al*., 1994; Young *et al*., 1999). Experimental evidences reveals dominant negative effect by some BBS mutations affecting other gene functioning (Zaghloul *et al*., 2010).

1.1.5 Pathophysciology of Disease

In case of BBS only a few symptoms are apparent at the time of birth they may be primary features or secondary features mostly polydactyly or renal problems are apparent at the time of birth (Forsythe & Beales, 2013). RCD mostly RP with macular involvement is the most frequent diagnostic sign triggering an examination for BBS (Baker & Beales, 2009). Rod photoreceptors die first, and then cone photoreceptors die afterwards (Datta *et al*., 2015). Electroretinography (ERG) shows changes during first two years of life (Baker & Beales, 2009). Another important clinical finding is obesity, which is prevalent in the BBS population at 72–86% (P. Beales *et al*., 1999; Tina Duelund Hjortshøj *et al*., 2010; Moore *et al*., 2005; Riise *et al*., 1997; Tobin & Beales, 2007). Hypogonadism, type 2 diabetes and developmental delay are also observed in BBS (P. Beales *et al*., 1999; Deveault *et al*., 2011;

Moore *et al*., 2005). Renal defects are major case of mortality and morbidity in BBS (Baker & Beales, 2009).

1.1.6 Signs and Symptoms of BBS

1: RCD, 2: Polydactyly, 3: Obesity, 4: Genital defects, 5: Renal abnormalities, 6: Learning problems are the six primary symptoms of BBS. Secondary signs include 1: developmental delay, 2: dental problems, 3: heart defects, 4: speech problem, 5: syndactyly or brachydactyly, 6: poor coordination or atoxia, 7: olfactory defects, 8: diabetes mellitus, 9: hypertension, 10: liver defects, 11: bronchial asthma, 12: otitis, 13: rhinitis, 14: craniofacial dismorphism (Baker & Beales, 2009; Forsythe & Beales, 2013; SA Khan *et al*., 2016; Shoemark, Dixon, Beales, & Hogg, 2015). BBS primary and secondary features are listed in fig1.1. Patients should be given a BBS diagnosis if they exhibit at least four out of the disease's six major symptoms. For the confirmation of BBS two secondary characters are required if only three primary traits are found. This diagnosis criteria primarily characterize BBS as a clinical entity and it cannot adequately describes a person with disease attenuated form or gene-specific expression of BBS (Estrada-Cuzcano *et al*., 2012; Pawlik *et al*., 2010). Because only polydactyly and kidney anomalies are frequently detected at or before birth, antenatal genetic screening guidelines that are more flexible are justified (Putoux *et al*., 2010). BBS clinical and genetic diagnosis is complicated because it has a clear phenotypic lap over with a few other ciliopathies, such as Joubert syndrome, Alström syndrome, McKusick-Kaufman syndrome, Senior-Loken syndrome or Meckel syndrome (Redin *et al*., 2012).

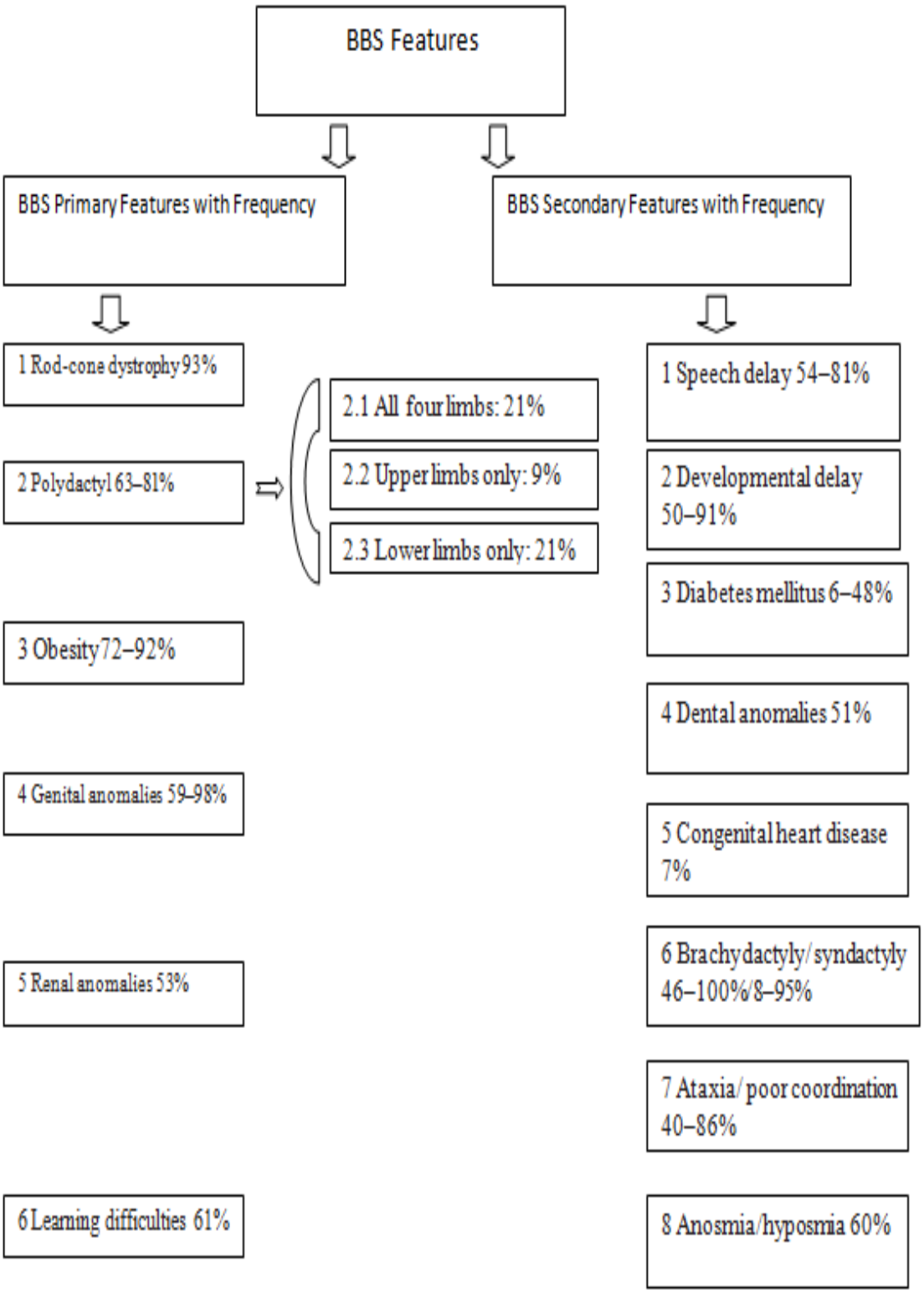

Fig.1.1: Primary and Secondary Symptoms of BBS.

1.1.6.1 Primary Signs of BBS

1.1.6.1.1 Rod-Cone Dystrophy (RCD)

RCD is observed in 94-100% of BBS patients and it has a prominent role in the diagnosis of BBS (Denniston *et al*., 2014; Green *et al*., 1989; Klein & Ammann, 1969; Niederlova, Modrak, Tsyklauri, Huranova, & Stepanek, 2019). There are reports that illustrates that due to malfunctioning of rod and cone cells severe progressive retinal degradation are caused. Night blindness has affected the people since they were young. In BBS patients, low vision over the first few years of life has been documented (Berezovsky *et al*., 2012). Rhodopsin is transported from inside of the rod cell to the outside of rod cell and this transportation is mainly performed by the BBSome complex and this is of prime importance for rod cell. Rhodopsin mislocalizes and accumulates in rod cells as a result of BBSome gene mutations as shown in Fig1.2. This results in the disruption of cellular homeostasis and the degradation of the photoreceptors (Mockel *et al*., 2011).

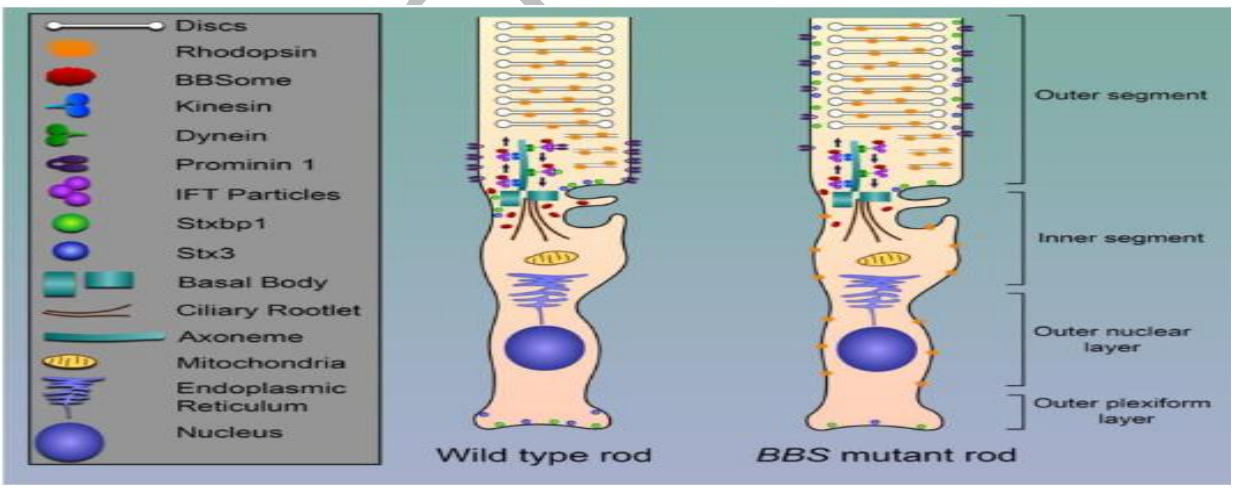

Fig.1.2: Mislocalization of proteins in mutant BBS rod. (Adopted from Weihbrecht, Goar *et al*, 2017)

Photoreceptor cells' outer segments (OSs) serve as particularize sensory cilia that develop into distinctive OS discs to offer a large surface area for maximum photon collection and effective visual transduction. Therefore, careful ciliary transport regulation is required to maintain the continuous restoration of about 10% of Oss. It has been proposed that Rab8 and the BBSome complex work together during intraflagellar transport (IFT) to mediate the ciliary transportation of the rhodopsin protein (Mockel *et al*., 2011). Knock-out mice models of BBS(2,3,4,6,14) genes proved induction of degeneration of photoreceptors due to mislocalization and buildup of rhodopsin in rod cell inner segment (IS) (Mockel *et al*., 2011). Only 5% of RP patients are exhibited with BBS syndrome (Haim, 1992; Klein & Ammann, 1969).

1.1.6.1.2 Obesity

Obesity is observed in 89% cases of BBS and it appears at the age of 2-3 years and it mostly effects abdomen and thorax (Guo & Rahmouni, 2011). Obesity may be produced due to1: BBS gene mutations, 2: altered Wnt and sonic hedgehog (Shh) signaling in differentiating preadipocytes, 3: deregulated appetite, 4: amended leptin resistance, 5: compromised leptin receptor signaling, 6: alterations in ciliated neurons' neuroendocrine signaling with fat storage tissues (Seo *et al*., 2009). BBS2 nucleotide polymorphism (rs4784675) is linked with adult obesity while BBS4(rs7178130) and BBS6(rs221667 and rs6108572) are linked with earlier-onset obesity in children and adult severe obesity (Benzinou *et al*., 2006).

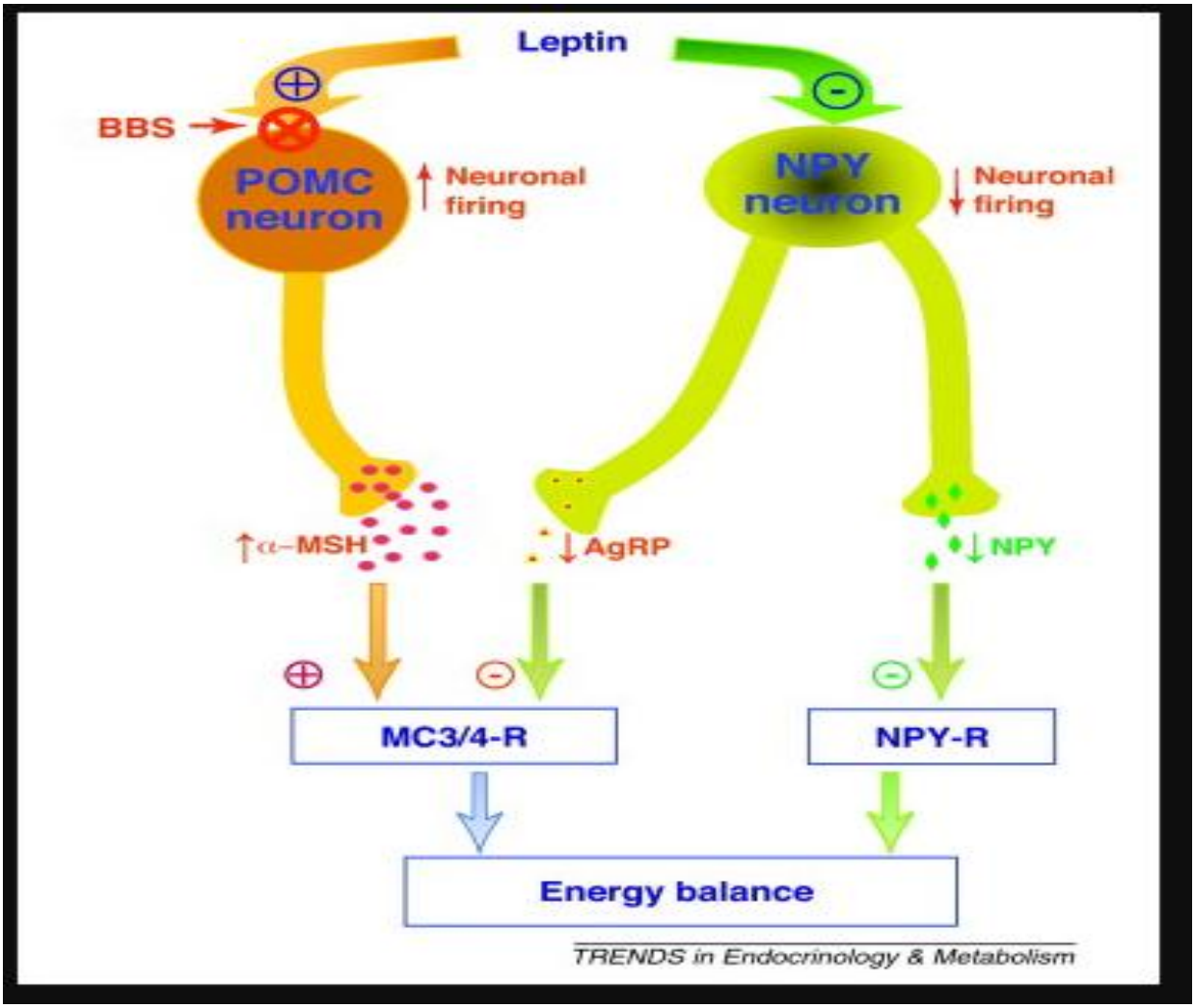

Fig.1.3: Role of Leptin in Energy Balance (Adopted from Guo and Rahmouni, 2011)

1.1.6.1.3 Polydactyly

In 79% of patients, postaxial polydactyly is frequently reported, toes polydactyly is more prevalent as compared to fingers polydactyly (Niederlova *et al*., 2019). Mesoaxial polydactyly has been linked to the *BBS17* gene and this is considered as negative regulator of the ciliary trade that is controlled by Shh (Sonic hedgehog) signaling and BBSome (Schaefer *et al*., 2014). Sonic hedgehog is activated through Smoothened

(Smo), Patched 1(Ptch 1) and then through Gli transcription factor which transduces the signal that explains the molecular association of ciliary proteins with formation of limb in BBS as Gli is involved in gene transcription. Ptch1 and Smo interact with BBS1 quite strongly (Forsythe & Beales, 2013). The BBSome's endogenous cargos are Smo and Ptch1, thus when mice lose their normal Bbs genes, Smo and Ptch1 build up in their cilia, which may cause polydactyl, the Shh response is also reduced quantitatively (Zhang, Seo, Bugge, Stone, & Sheffield, 2012).

1.1.6.1.4 Hypogonadism

The incidence of hypogonadism and genitourinary abnormalities is 59% (Niederlova *et al*., 2019).At puberty, hypogonadism may become noticeable. Male anomalies can include small-volume testicles, cryptorchidism, and hypogonadotropic hypogonadism, among others anomalies (Mujahid *et al*., 2018) . Female anatomical anomalies include atresia or hypoplasia of the uterus, fallopian tubes, ovary, or vagina (Niederlova, Modrak *et al*. 2019. Both sexes may possess children's but fertility rate is low (Forsythe & Beales, 2013). Knock-out mice for *Bbs2/Bbs4* genes were not producing progeny as they were lacking flagellated sperm and it depicts the importance of these genes proteins in spermatogenesis as in flagella formation (Mykytyn *et al*., 2004). Studies conducted in vitro reveal that BBS4 resides to the centriolar satellites of centrosomes and basal bodies, which are required to attract PCM1 to centrosomal satellites. BBS4 likely does this by functioning as an adapter between PCM1 and the dynein-dynactin motor complex. PCM1 and its related protein were mislocalized as a result of BBS4 null/truncating mutations, which led to significant microtubule disarray. This interferes with centrosome and basal body function, which in turn affects ciliary function (J. C. Kim *et al*., 2004).

1.1.6.1.5 Developmental Delay

One of the ciliopathy characteristic that is still the poorly understood is developmental delay. Emotional instability, flawed reasoning, and limited attention span are some behavioral alterations that have been noticed in these patients. Behavioral problems with a similar pattern have been seen in *Bbs* knockout mice. Although *BBS3/Arl6* being present in neural tissues, it is yet unclear how BBS proteins results in cognitive impairment (Zaghloul & Katsanis, 2009). Planar cell polarity (PCP) and Shh signaling are influenced by BBS proteins and it raises the possibility that they play a role in the proliferation and migration of developing neuronal populations (Louvi & Grove, 2011).

1.1.6.1.6 Renal Defects

Kidney disease in BBS patients is a common reason for morbidity and mortality and it has been reported in 52% of BBS cases (Niederlova *et al*., 2019). Most of cases of BBS with renal disorders are diagnosed at the age of five (Forsythe *et al*., 2017). This disease varies from chronic glomerulonephritis, cystic tubular disease ,defective tubular concentrating ability and urinary tract malformations that results in morbidity and mortality (P. Beales *et al*., 1999). Renal pathologies in BBS patients are explained by mice models that depicted the structural defects in cilia of renal tubes results in faulty cilia assembly or faulty maintenance of ciliary length. The character of Wnt planar cell polarity (Wnt PCP) pathway that is upstream of mammalian target of rapamycin (mTOR) has been exposed in the kidney phenotype. Zebra fish models show that the mTOR signaling inhibitor rapamycin can restore the kidney cysts in BBS mutant embryos(Cardenas-Rodriguez *et al*., 2013). Proteins found in the kidney interact with BBS proteins (BBS1, BBS2, BBS4, and BBS7).

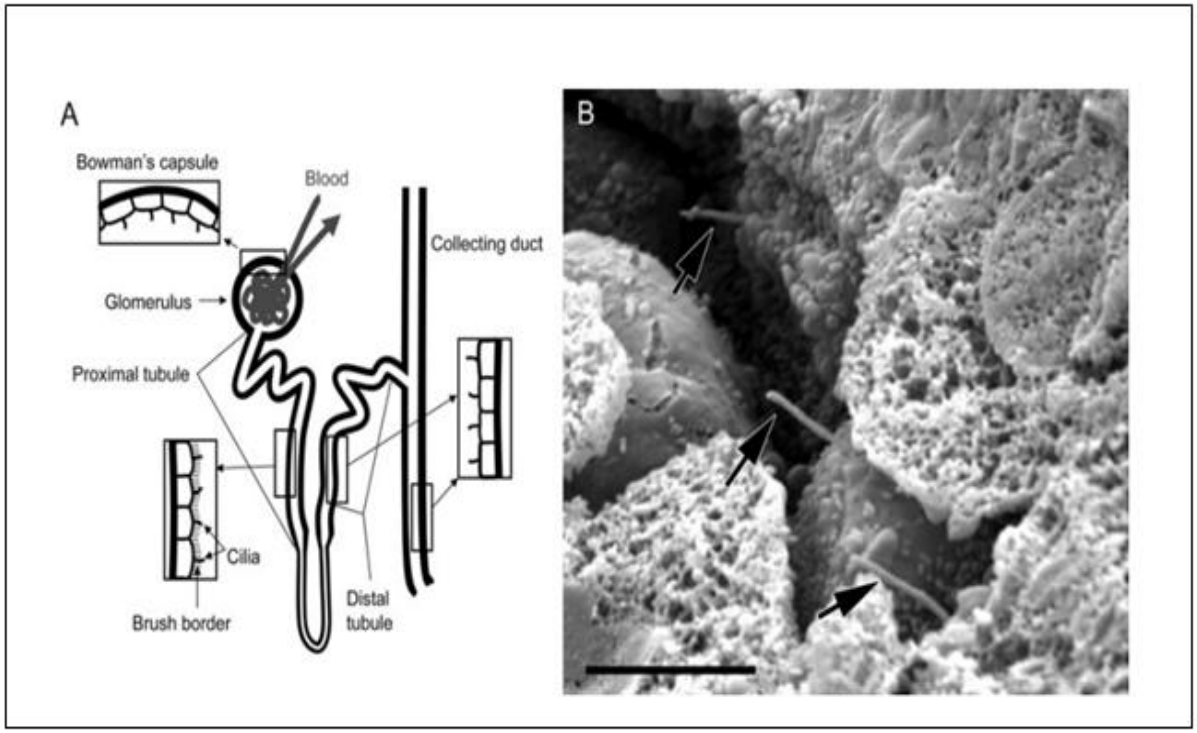

Fig.1.4: (A) Localization of cilia in nephron. (B) Scanning electron micrograph showing primary cilia in nephron. (Adopted from Deane and Ricardo, 2012)

The renal cells in BBS individuals with nearly normal kidney function are mostly deprived of cilia and display a normal cell cycle but they have inactive intracellular water absorption mechanism. These patients also lacked renal cysts and urine concentration problem (Marion *et al*., 2011). Vasopressin receptor has chemosensory role in renal epithelial cells and it is also implicated in renal failure of BBS patients (Raychowdhury *et al*., 2009). By controlling the expression of genes related to the epithelial-to-mesenchymal transition, fibrosis, and apoptosis, the kidney's abundantly present BBS1 protein has shown to be involved in the transport of GLIS2, which is necessary for sustaining renal functioning (Y. H. Kim *et al*., 2013).

1.1.6.2 Secondary Minor Features

Minor symptoms of BBS affect various organ and systems. Minor symptoms include nervous system defects, gastrointestinal defects, endocrine glands defects, cardiovascular defects, musculoskeletal defects and cutaneous defects (Forsythe & Beales, 2013). Ataxia, poor coordination with mild hypertonia of all four extremities, speech defects, behavioral defects, psychiatric defects and seizures are observed in BBS patients with neurodevolpmental defects. Brachycephaly and macrocephaly are the most commonly reported traits in BBS other traits includes, large ears, narrow forehead, deep and widely set eyes, malar flattening, depressed nasal bridge, retrognathia, smooth and long philtrum, short narrow and downslanted palpebral fissures. Dysmorphism in BBS is also reported but it is not specific. Dental defects are reported in 50% cases of BBS (Forsythe & Beales, 2013). Defects in olfactory bulb or olfactory cilia are related to hyposomia/ansomia (Braun *et al*., 2016). Cardiovascular defects are reported in 29% BBS cases (Niederlova *et al*., 2019). Different gastrointestinal and endocrine/metabolic abnormalities are reported in BBS cases (RaeLynn Forsyth & Gunay-Aygun, 2020). Metabolic defects are reported in 54.3% BBS patients. Hyperlipidemia (usually hypertriglyceridemia), obesity, elevated fasting plasma glucose with or without type 2 diabetes mellitus and insulin resistance are reported in 15.8% of BBS patients and polycystic ovarian syndrome in 14.7%, and subclinical hypothyroidism in 19.4% of BBS patients is reported (RaeLynn Forsyth & Gunay-Aygun, 2020; Mujahid *et al*., 2018; Tsyklauri *et al*., 2021).

1.1.6.3 Involvement of BBS Genes in other Syndromes

Mutations in BBS causing genes are recently known to be involved in non-syndromic retinal degenerations and in other ciliopathies such as Leber Congenital Amaurosis (LCA), Joubert Syndrome and Senior-Loken Syndrome (Weihbrecht *et al*., 2017).

1.8. Biology of Disease

Cilia projects from apical surface of most vertebrate cells and these has two types motile and immotile cilia, motile cilia has 9+2 microtubule organization while immotile cilia has 9+0 microtubule organization as shown in fig1.5 (Waters & Beales, 2011). Immotile cilia are involved in signaling and perform a sensory function (Baker & Beales, 2009; Tobin & Beales, 2007; Waters & Beales, 2011). Defective motile cilia may result ciliary dyskinesias exhibiting infertility, left-right asymmetry and bronchiectasis. Immotile cilia have similarity with motile cilia but these are without central pair and these defective immotile cilia results in RP, learning difficulties, polydactyly, sinus inversus, kidney, liver and pancreatic defects. Immotile defective cilia are involved in cause of BBS syndrome as shown in fig 1.6 (Baker & Beales, 2009; Tobin & Beales, 2007; Waters & Beales, 2011).

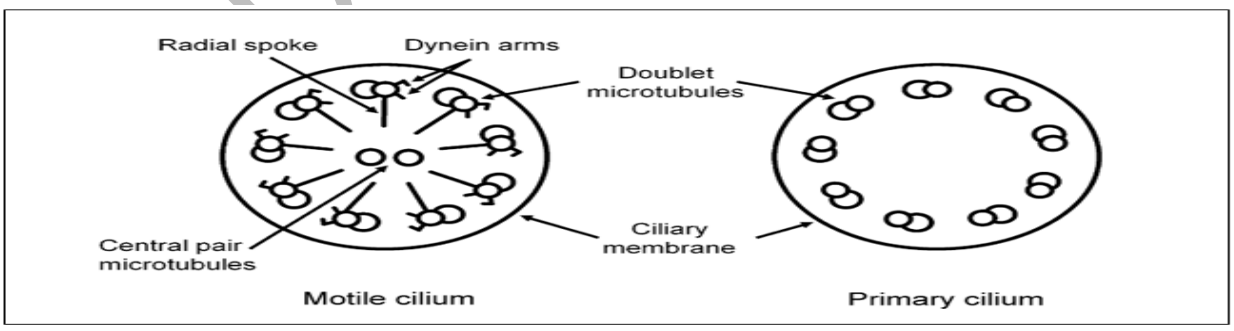

Fig.1.5: Structure of motile cilia and primary cilia. (Adopted from Deane and Ricardo, 2012)

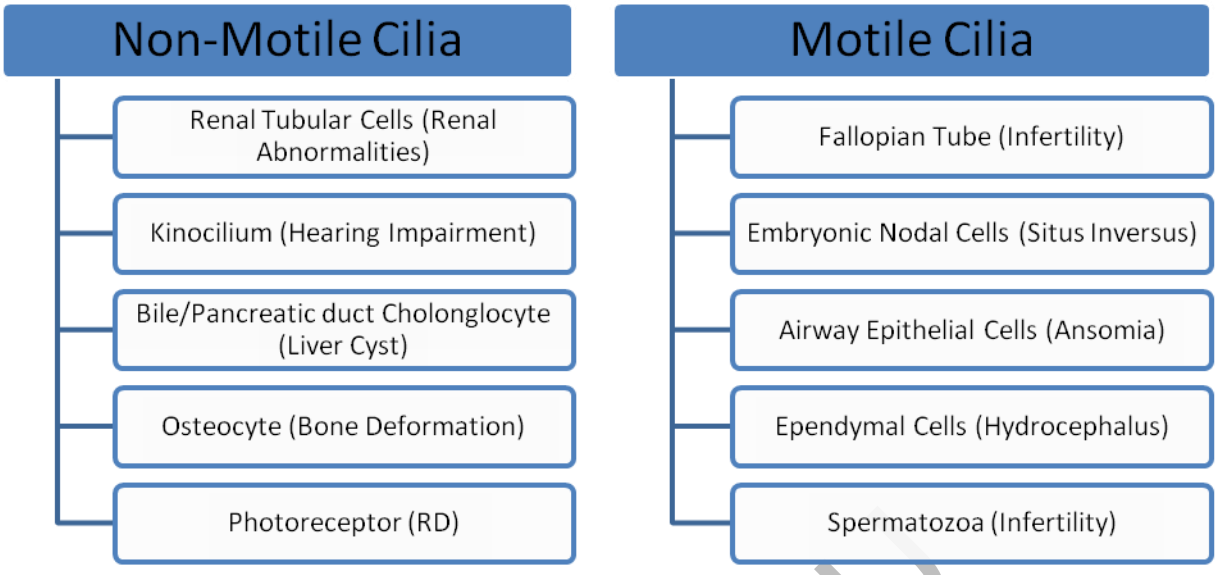

Fig.1.6: Defects in non-motile cilia and motile cilia results in different disorders.

Basal body serves as a hub for organizing microtubules, and the cilium is tethered in basal body. Ciliogenesis and maintenance of cilium is done by the basal body in conjugation with BBSome and this process is regulated by the chaperon complex and members of Rab family (Waters & Beales, 2011). Cellular bidirectional movement of particles plays an important role in maintenance and formation of cilium. All BBS genes have role in cilia functioning, (BBS1,2,4,5,7,8,9,17,18) are involved in BBSome formation while (BBS6,10,12) are involved in formation of chaperonin complex and (BBS13,14,15,16) are involved in basal body formation. (BBS3,11,19,20 and NPHP1) has biological role related to cilia functioning (Tayeh *et al*., 2008). No phenotypic differences are observed in patients with gene mutations in BBSome and chaperonin complex (Forsythe & Beales, 2013). Pre-adipocytes are containing primary cilia transiently and receptors for Wnt and hedgehog pathway are present in primary cilia and these are important for development. The repression of BBS10 and BBS12 promotes adipogenesis by disruption of Wnt signaling (Marion *et al*., 2009). It is believed that rod-cone dystrophy results from aberrant trafficking across the

damaged modified cilia linking the inner and outer segments of photoreceptors, and this results in apoptosis. In fig 1.7 schematic representations of primary cilium and its role in signaling is depicted. Disruption in different signaling pathways leads to BBS (Mockel *et al*., 2011; Nishimura *et al*., 2004; Sheffield, 2010).

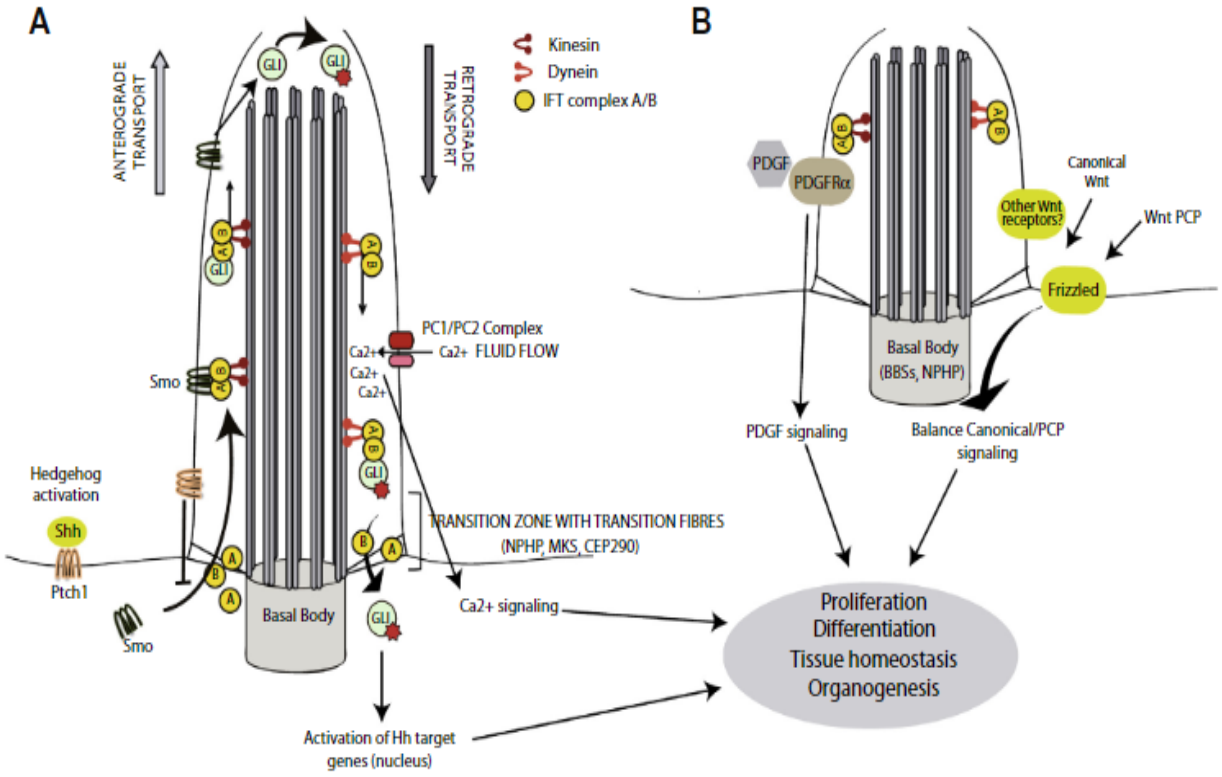

Fig.1.7: Schematic representation of cilium and its role in signal transduction. (A) Mechanism of gene transcription by hedgehog activation. (B) Proteins regulating the Wnt signaling. (Adopted from Novas, Cardenas-Rodriguez *et al*, 2015)

Ciliary proteins are sorted in and out of cilium by BBSome that is a conserved protein complex in ciliated organisms. The G-protein coupled receptors (GPCRs) that are involved in sonic hedgehog signaling especially Smo and GPR161 and neuronal receptors NPY2R, MCHR1, SSTR3 and D1R are main cargo of BBSome (Berbari, Lewis, Bishop, Askwith, & Mykytyn, 2008; Klink *et al*., 2017; Loktev & Jackson, 2013; van Dam *et al*., 2013; Vickers, 2017; Zhang *et al*., 2013; Zhang, Seo, *et al*., 2012). BBSome may also play a role in the

transport of the leptin receptor to the cell membrane, according to a recent theory(Guo *et al*., 2016; Seo *et al*., 2009). According to research done on lower eukaryotes, the BBSome is also capable of carrying cargo without transmembrane domains (Liu & Lechtreck, 2018).

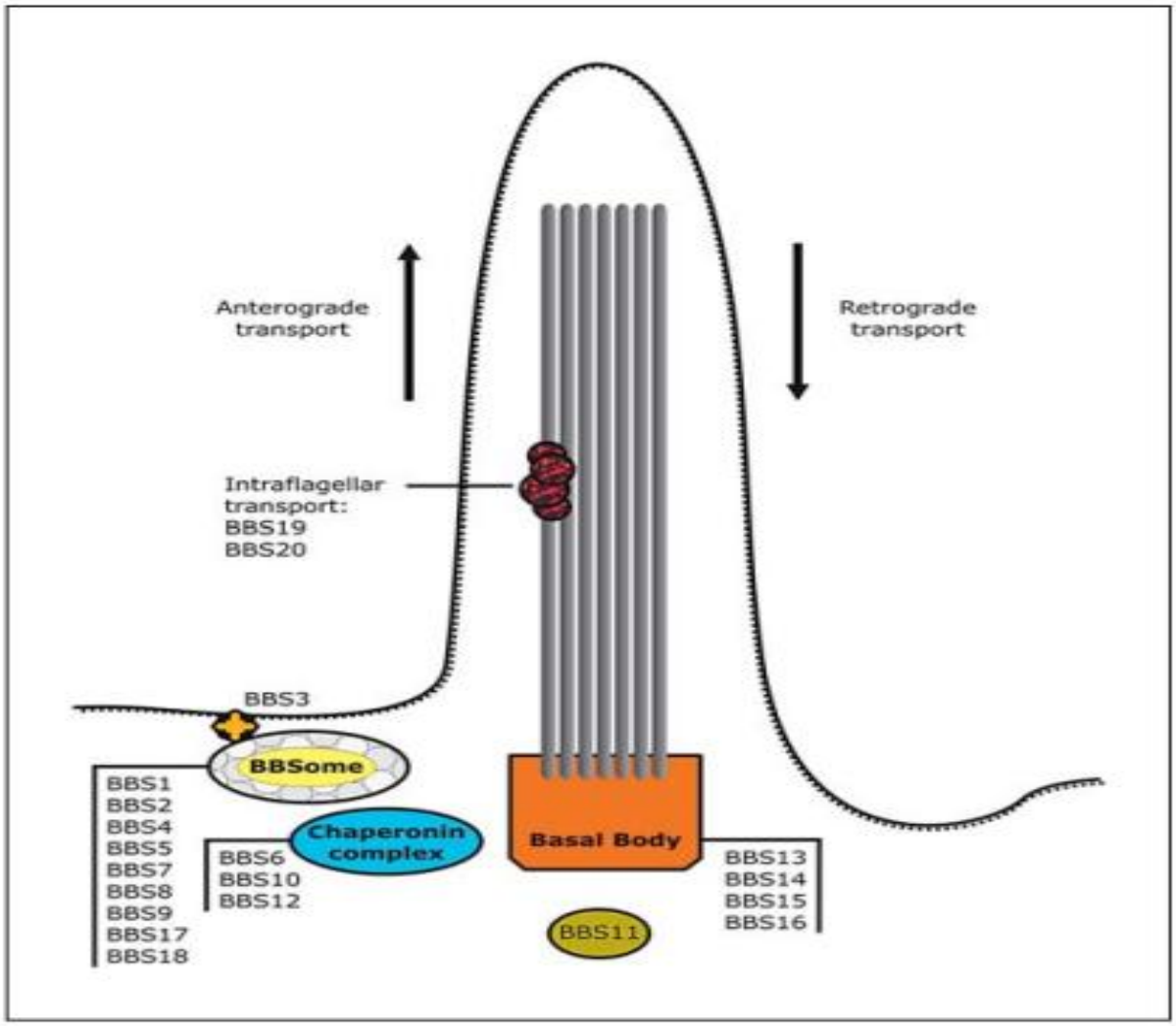

Fig.1.8: BBS proteins and BBSome (Adopted from (Suspitsin and Imyanitov, 2016)

The defects of different organs in BBS due to defiency of BBSome are poorly understood. Retinal Detachment (RD) is considered to be caused by the defects in photosensitive GPCR rhodopsin that causes photoreceptor degeneration but in an in vitro study rhodopsin was not found as BBSome cargo (Abd-El-Barr *et al*., 2007; Klink *et al*.,

2017; Nishimura *et al*., 2004). The buildup of intrinsic proteins in the outer segments of photoreceptors has also been suggested as a possible cause of photoreceptor atrophy in BBS patients and animal models (Datta *et al*., 2015). Defective hedgehog signaling is involved in PD and dental defects, defective neuronal GPCRs are involved in developmental delay and defective neurological appetite control that is involved in obesity (Liu & Lechtreck, 2018; Loktev & Jackson, 2013; McIntyre, Hege, & Berbari, 2016; Zhang, Seo, *et al*., 2012). BBSome is proposed to be involved in ciliary localization and functioning of polycystin (PC) 1 and 2 and mislocalization of these results in kidney diseases (Su *et al*., 2014; Tobin & Beales, 2007; Xu *et al*., 2015).

1.9. Genetics of Baedet-Biedl Syndrome

Presently 26 genes are investigated to be involved in BBS syndrome. A minimum of 24 genes are proven to have functional role in primary cilium and loss-of-function (LOF) mutations in these genes contribute to BBS, an autosomal recessive syndrome. These genes are (*BBS1-21, NPHP1, IFT74 and SCAPER*). Most BBS cases have mutations in those eight genes that are involved in the formation of BBSome protein complex these genes are (*BBS1, BBS2, BBS4, BBS5, BBS7, BBS8, BBS9 and BBS18*). Genes that encode chaperonins that aid in the assembly of the BBSome are mutated in the second largest cohort of BBS patients these are *BBS6/MKKS*. A genetic variation in *BBS3/ARL6*, a GTPase supporting BBSome function, is present in the third most frequent subset of patients. Mutations in other BBS genes are rare and these are mostly involved in origination of other ciliopathies. All BBS genes have role in cilia functioning, (*BBS1, 2, 4, 5, 7, 8, 9, 17, 18*) are involved in BBSome formation while (*BBS6, 10, 12*) are involved in formation of chaperonin complex and

(*BBS13, 14, 15, 16*) are involved in basal body formation. (*BBS3, 11, 19, 20 and NPHP1*) has biological role related to cilia functioning.

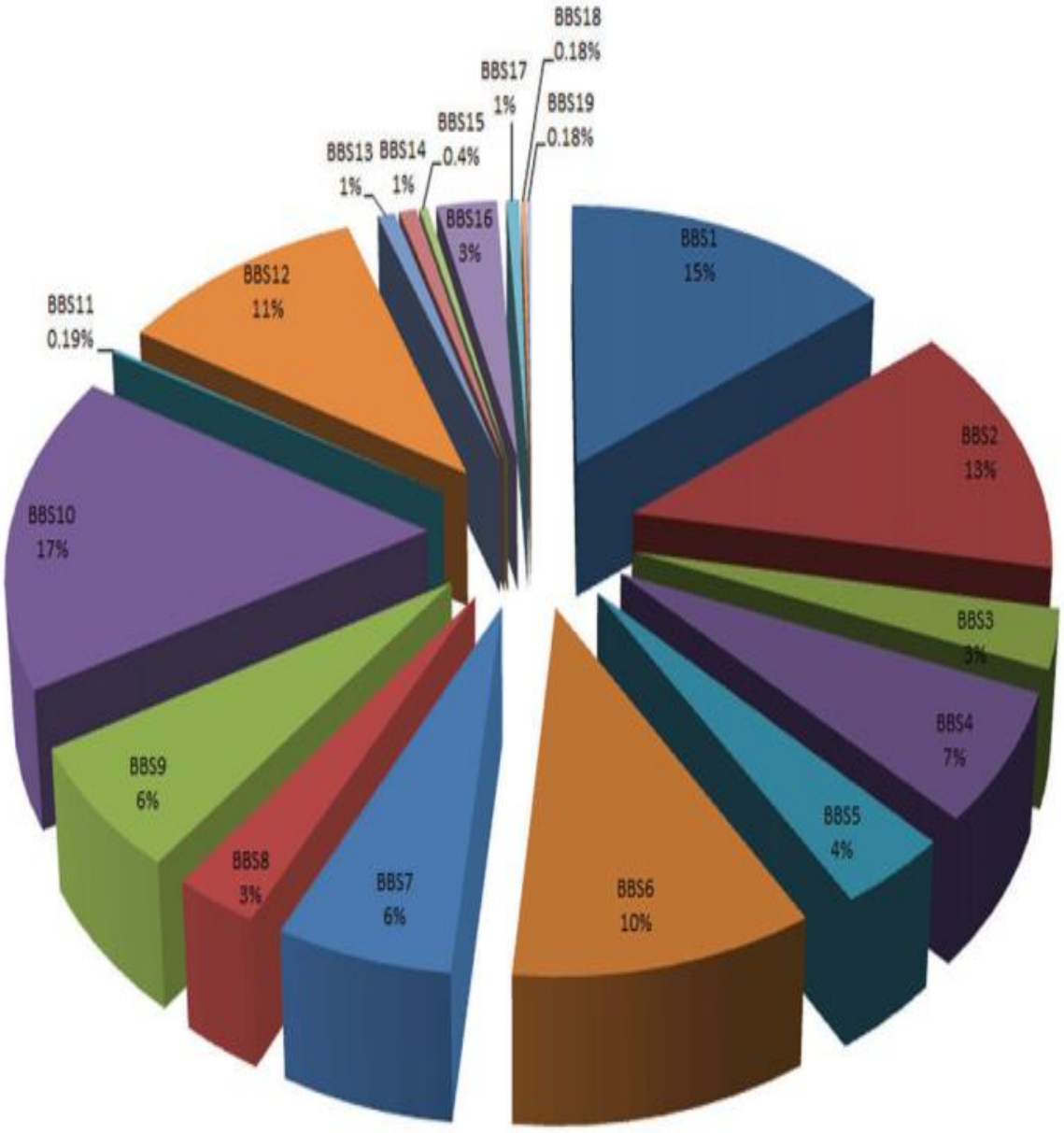

Fig.1.9: Involvement of different BBS genes toward total mutational load in 2016. (Adopted from Khan, Muhammad *et al*, 2016)

Table 1.1: Description of Genes involved in Bardet-Biedl Syndrome

S.No	GENE	SYMBOL OF GENE	GENE FULL NAME	CHROMOSOMAL LOCATION
1	*BBS1*	*BBS1*	Bardet-Biedl syndrome 1	11q13.2
2	*BBS2*	*BBS2*	Bardet-Biedl syndrome 2	16q13
3	*BBS3*	*ARL6*	ADP ribosylation factor like GTPase 6	3q11.2
4	*BBS4*	*BBS4*	Bardet-Biedl syndrome 4	15q24.1
5	*BBS5*	*BBS5*	Bardet-Biedl syndrome 5	2q31.1
6	*BBS6*	*MKKS*	MKKS centrosomal shuttling protein	20p12.2
7	*BBS7*	*BBS7*	Bardet-Biedl syndrome 7	4q27
8	*BBS8*	*TTC8*	Tetratricopeptide repeat domain 8	14q31.3
9	*BBS9*	*BBS9*	Bardet-Biedl syndrome 9	7p14.3
10	*BBS10*	*BBS10*	Bardet-Biedl syndrome 10	12q21.2
11	*BBS11*	*TRIM32*	Tripartite motif containing 32	9q33.1
12	*BBS12*	*BBS12*	Bardet-Biedl syndrome 12	4q27
13	*BBS13*	*MKS1*	MKS transition zone complex subunit 1	17q22
14	*BBS14*	*CEP290*	Centrosomal protein 290	12q21.32
15	*BBS15*	*WDPCP*	WD repeat containing planar cell polarity effectors	2p15
16	*BBS16*	*SDCCAG8*	SHH signaling and ciliogenesis regulator SDCCAG8	1q43-q44
17	*BBS17*	*LZTFL1*	Leucine zipper transcription factor like 1	3p21.31
18	*BBS18*	*BBIP1*	BBSome interacting protein 1	10q25.2
19	*BBS19*	*IFT27*	Intraflagellar transport 27	22q12.3
20	*BBS20*	*IFT74*	Intraflagellar transport 74	9p21.2
21	*BBS21*	*C8orf37*	Chromosome 8 open reading frame 37	8q22.1
22	*BBS22*	*SCLT1*	Sodium channel and clathrin linker 1	4q28.2
23	*BBS23*	*NPHP1*	Nephrocystin 1	2q13
24	*BBS24*	*SCAPER*	S-phase cyclin A associated protein in the ER	15q24.3

Table 1.2: Function of BBS genes their Localization, Tissue spacifity and Proteins encoded by these Genes

S.No	GENE	GENE GROUPS	PROTEIN	LOCALIZATION	TISSUE SPACIFITY	GENE FUNCTION
1	*BBS1*	BBSome	Bardet-Biedl syndrome 1 protein	Cilium, Basal body	Least tissue specific	Formation of BBSome complex formation
2	*BBS2*	BBSome	Bardet-Biedl syndrome 2 protein	Cilium, Basal body	Least tissue specific	Formation of BBSome complex formation
3	*ARL6*	ARF GTPase family	ADP-ribosylation factor-like protein 6	Cilium, Basal body, Transition zone	Least tissue specific	GTPase; facilitates vesicular and interciliary trafficking
4	*BBS4*	Tetratricopeptide repeat domain containing BBSome	Bardet-Biedl syndrome 4 protein	Cilium, Basal body	Least tissue specific	Formation of BBSome complex formation
5	*BBS5*	BBSome	Bardet-Biedl syndrome 5 protein	Basal body	Least tissue specific	Formation of BBSome complex formation
6	*MKKS*	Chaperonins	McKusick-Kaufman/Bardet-Biedl syndrome putative chaperonin	Cytosol, Basal body	Least tissue specific	Formation of chaperonin complex
7	*BBS7*	BBSome	Bardet-Biedl syndrome 7 protein	Cilium, Basal body	Least tissue specific	Formation of BBSome complex formation
8	*TTC8*	Tetratricopeptide repeat domain containing BBSome	Tetratricopeptide repeat protein 8	Cilium, Basal body, IFT	Least tissue specific	Formation of BBSome complex formation
9	*BBS9*	BBSome	Protein PTHB1	Cilium	Least tissue specific	Formation of BBSome complex formation
10	*BBS10*		Bardet-Biedl syndrome 10 protein	Basal body	Least tissue specific	Formation of chaperonin complex
11	*TRIM32*	Tripartite motif containing Ring finger proteins	E3 ubiquitin-protein ligase TRIM32	Intermediate filament	Least tissue specific	E3 ubiquitin complex
12	*BBS12*	Chaperonins	Bardet-Biedl syndrome 12 protein	Basal body	Least tissue specific	Chaperonin complex

13	*MKS1*	B9 domain containing MKS complex	Meckel syndrome type 1 protein	Basal body	Least tissue specific	Epithelial morphogenesis and ciliary formation
14	*CEP290*	MKS complex	Centrosomal protein of 290kDa(Cep290)	Centrosome, Basal body	Least tissue specific	Microtubule associated protein transport
15	*WDPCP*	Ciliogenesis and planar polarity effector complex subunits	WD repeat-contaning and planar cell polarity effector protein fritz homolog (Hfrtz)	Axoneme, Plasma membrane, Cytosol	Least tissue specific	Regulation of cell polarity and directional cell migration
16	*SDCCAG8*	MicroRNA protein coding host genes	Serologically defined colon cancer antigen 8	Centriole, Basal body, Transition zone	Least tissue specific	Regulation of cell polarity
17	*LZTFL1*		Leucine zipper transcription factor like protein	Cilium, Basal body	Highly tissue specific especially(lymphoid tissue)	Negative regulation of BBSome trafficking
18	*BBIP1*	BBSome	BBSome interacting protein 1	Cytoplasm, Cytosol	Highly tissue specific especially(testis)	Formation of BBSome complex formation
19	*IFT27*	IFT-B1 complex RAB, member RAS oncogene GTPase	Intraflagellar transport protein 27 homolog	Cilium, Basal body, IFT	Least tissue specific	Intraflagellar transport
20	*IFT74*	IFT-B1 complex	Intraflagellar transport protein 74 homolog	Cilium, Basal body, IFT	Least tissue specific	Ciliogenesis and length control
21	*C8orf37*		Protein C8orf37	Basal body, Ciliary root	Least tissue specific	Maintains photoreceptor outer segment membrane
22	*SCLT1*		Sodium channel and clathrin linker 1	Centriole	Least tissue specific	
23	*NPHP1*	NPHP complex	Nephrocystin-1	Transition zone	Highly tissue specific especially(skeletal muscle)	
24	*SCAPER*	Zinc finger C2H2-type	S phase cyclin A associated protein in the endoplasmic reticulum (S phase cyclin A associated protein in the ER)	Endoplasmic Reticulum	Least tissue specific	Cilia length

1.3.1. Bardet-Biedl syndrome-1 (MIM 209900)

BBS1 syndrome is caused by *BBS1* gene that is located on chromosome 11 long arm and its position is (11q13.2) and its expression is mostly reported in retina, cardiac tissues, pancreatic tissues, skeletal tissues, testes and in fetal tissues. In kidney highest expression of *BBS1* gene is reported (Mykytyn *et al*., 2002). *BBS1* gene consists of 17 coding exons and its size is almost 23 kb. The BBS1 gene's normal protein has no similarity with known BBS proteins, only a fragment at N-terminal end consisting of beta-propeller domain shows similarity with *BBS2* and *BBS7*. Different stages of various signaling pathways are affected differently by the allelic variations of the *BBS1* gene. *BBS1* has a major role in leptin receptor signaling pathway and in biogenesis of ciliary membrane (Blacque *et al*., 2004; Loktev *et al*., 2008; Mykytyn *et al*., 2003; Nachury *et al*., 2007).

1.3.2. Bardet-Biedl syndrome-2 (MIM 615981)

BBS2 syndrome is caused by the *BBS2* gene and its location is on chromosome 16 and its position on chromosome 16 is (16q21). *BBS2* expression is mostly reported in thyroid glands, adrenal glands, kidney and brain (Nishimura *et al*., 2004). *BBS2* consist of 17 exons that encodes a 721 amino acid protein. *BBS2* is a part of BBSome and it has role in ciliary membrane biogenesis. *BBS2* pathogenic variants are associated with obesity, retinopathy and substandard social functions. *BBS2* and *BBS4* deficient neuron does not have ciliary localization of *MCHR1* that is involved in feeding regulation and *SSTR3.* The normal protein coded by the *BBS2* gene shows no homology to investigated proteins, only a fragment at N-terminal end consisting of beta-propeller domain shows similarity with *BBS1* and *BBS7* (Berbari *et al*., 2008; Nishimura *et al*., 2004).

1.3.3. Bardet-Biedl syndrome-3 (MIM 600151)

ARL6 is the causative gene for BBS3 syndrome and its location is on chromosome 3 and its position on chromosome is (3p12-p13). *ARL6* contains 9 exons and ADP-ribosylation factor- like protein 6 of 186 amino acid is encoded by this gene (Ingley *et al*., 1999). ADP-ribosylation factor (ARF) family is a part of RAS super family and *ARL6* consist of conserved features of ARF family that are N-terminal myristoylation site, a hydrophobic alpha helix and a GTP-binding site. *ARL6* has an important role in hemopoietic development (Ingley *et al*., 1999; Moss & Vaughan, 1995; Pasqualato, Renault, & Cherfils, 2002; Takada, Iida, Sasaki, Taira, & Kimura, 2004).

1.3.4. Bardet-Biedl syndrome-4 (MIM 615982)

BBS4 is the causative gene for BBS4 syndrome and its location is on chromosome 15 and its position on chromosome is (15q22.3-q23). *BBS4* size is almost 52 kb consisting of 16 exons having open reading frame of 519 codons encoding protein of 519 amino acids. At least ten TPR domains, which are present in *BBS4*, are considered to be involved in protein-protein interactions. It is found at centriolar satellites of the centrosomes and the basal bodies of primary cilia. It might work as an adapter protein to help load materials onto the dynein dynactin molecular motor for microtubule-dependent intracellular transportation in the cilium or cytosol (J. C. Kim *et al*., 2004). Pathogenic allelic variants of *BBS4* are thought to be involved in RD, obesity, sperm flagellation defects and olfactory defects. *BBS4* silencing in cultured cells leads to apoptotic cell death, arrest of cell division and deanchoring of microtubules. *BBS2* and *BBS4* deficient neurons does not have ciliary localization of *MCHR1* that is involved in feeding regulation and *SSTR3* (Fattahi *et al*., 2014; Mykytyn *et al*., 2004; Pasqualato *et al*., 2002).

1.3.5. Bardet-Biedl syndrome-5 (MIM 615983)

BBS5 is responsible gene for BBS5 and its location is on chromosome 2 and its position on chromosome is (2q31.1). *BBS5* consists of twelve exons and the protein encoded by it, is implicated in flagella and cilia origination and has two pleckstrin homology domains that bind to phosphoinositide. Ciliogenesis is inhibited when the production of phosphoinositide is inhibited this depicts a linkage between the IFT machinery and ciliary membrane (Li *et al*., 2004).

1.3.6. Bardet-Biedl syndrome-6 (MIM 605231)

MKKS is the causative gene for BBS6 syndrome and its location is on chromosome 20 and its position on chromosome is (20p12). *MKKS* contains 6 exons that encodes a 570 amino acid protein that has homology to chaperonin family members and its role is in reproductive, cardiac and limbs systems. BBS6 protein has role in cytokinesis (Slavotinek *et al*., 2000).

1.3.7. Bardet-Biedl syndrome-7 (MIM 615984)

BBS7 is the causative gene for BBS7 syndrome and its location is on chromosome 4 and its position on chromosome is (4q27). BBS7 consist of nineteen exons that encodes a protein of 672 amino acid. BBS7 has similarity of 252 amino acid with BBS2 region from 147 to 398 residues. There is a structural link between (*BBS1, 2* and *BBS7)* which indicates that these genes results in proteins of distinctive subfamily and mutations in these genes causes similar clinical signs (Badano *et al*., 2003).

1.3.8. Bardet-Biedl syndrome-8 (MIM 615985)

TTC8 is the causative gene for BBS8 syndrome and its location is on chromosome 14 and its position on chromosome is (14q32.1). *TTC8* consists of fifteen exons and a 531 amino acid protein is encoded by it. BBS8 is also a part of BBSome complex and it has similarity with BBS4 protein due to eight TPR domains that are implicated in protein-protein interactions. In cultured ciliated cells BBS protein is located at basal body and centrosome (Ansley *et al*., 2003).

1.3.9. Bardet-Biedl syndrome-9 (MIM615986)

PTHB1 gene is responsible for BBS9 and its locality is on chromosome 7 and its position on chromosome is (7p14). *PTHB1* contains 23 exons and a 887 amino acid protein is encoded by it. This gene is mostly expressed in brain, kidney, liver, placenta, lungs, fetal kidney, skeletal muscles and adult heart and it has role in BBSome formation and ciliogenesis (Nachury *et al*., 2007; Vernon *et al*., 2003).

1.3.10. Bardet-Biedl syndrome-10 (MIM 615987)

C12ORF58/BBS10 gene is responsible for BBS10 syndrome and its locality is on chromosome 12 and its position on chromosome is (12q21.2). *BBS10* contains 2 exons and a 723 amino acid protein is encoded by it. BBS10 belongs from type II chaperonin subfamily and it consist of apical, equatorial and intermediate domains that are considered as chaperonin domain structural organization. It also consists of flexible protruding regions that are specific to type II chaperonin. BBS10 also possess ATP hydrolytic domain that is lacking in BBS6 and it is considered as playing an important role in enzyme activity (Marion *et al*., 2009; Nachury *et al*., 2007).

1.3.11. Bardet-Biedl syndrome-11 (MIM 615988)

TRIM32 is the causative gene for BBS11 syndrome and its location is on chromosome 9 and its position on chromosome is (9q33.1). BBS11is mostly expressed in adipose tissue and it contains 2 exons that encode a652 amino acid protein. TRIM32 belongs to TRIM family and it consist of B-box, RING finger and coiled-coiled motif. It also contains 5 C-terminal NHL repeats. TRIM32 possesses E3 ubiquitin ligase activity that binds to myosin and ubiquitinase actin depicting the role of TRIM32 in regulators of cytoskeleton. This gene's mutations have been linked to characteristics associated with muscular dystrophy (Chiang *et al*., 2006; Kudryashova, Wu, Havton, & Spencer, 2009; Locke, Tinsley, Benson, & Blake, 2009).

1.3.12. Bardet-Biedl syndrome-12 (MIM 615989)

BBS12 is the causative gene for BBS12 syndrome and its location is on chromosome 4 and its position on chromosome is (4q27). *BBS12* contains 2 exons and a 710 amino acid protein is encoded by it. BBS12 belongs to a family of vertebrate-specific chaperonin-like sequences that also includes BBS10 and BBS6, as well as the group II chaperonins. It consists of apical, equatorial and intermediate domains that are considered as chaperonin domain structural organization. The intermediate and equatorial domains of BBS12 additionally contain an additional five distinct insertion sequences. It also consists of flexible protruding regions that are specific to type II chaperonin that are also noted in BBS6 and BBS10 (Stoetzel *et al*., 2007).

1.3.13. Bardet-Biedl syndrome-13 (MIM 615990)

MKS1 (Meckel-Gruber syndrome 1) is responsible for BBS13 syndrome and its location is on chromosome 17 and its position on chromosome is (17q23). *BBS13* contains 18

exons and a 559 amino acid protein is encoded by it. MKS1 contains a B9 conserved domain and it is mostly located to basal body or primary cilium or both. This protein has role in ciliary function as these proteins contains X-box consensus sequence within promoter regions and these sequences are thought to be documented and controlled by the daf-19 or rfx family of transcription factors (Bialas *et al*., 2009; Dawe *et al*., 2007; Kyttälä *et al*., 2006; Tammachote *et al*., 2009; Williams *et al*., 2011).

1.3.14. Bardet-Biedl syndrome-14 (MIM 615991)

CEP290 (centrosomal protein 290 kDa) is the responsible for BBS14 syndrome and its location is on chromosome 12 and its position on chromosome is (12q21.3). *BBS14* contains 54 exons and a 2481 amino acid protein is encoded by it. Cerebellar granular neurons mostly express CEP290. It is restricted to the connecting cilium of photoreceptor cells as well as the centrosome and basal bodies of cilia in renal epithelial cells. Recent research has shown that the ciliary protein CEP290 interacts with the centriolar satellite protein PCM-1. In a microtubule-dependent manner, CEP290 binds to PCM-1 to form a complex that localizes to the centriolar satellites. It appears that CEP290 is necessary for the cytoplasmic microtubules to remain intact. Additionally, CEP290 and PCM-1 play a crucial role in ciliogenesis and in directing the small GTPase Rab8 to the ciliary membrane. Numerous ciliopathies, including BBS, have been associated to CEP290 mutations (J. Kim, Krishnaswami, & Gleeson, 2008; Sayer *et al*., 2006; Valente *et al*., 2006).

1.3.15. Bardet-Biedl syndrome-15 (MIM 615992)

C2ORF86/WDPCP is responsible for BBS15 syndrome and its location is on chromosome 2 and its position on chromosome is (2P15). *BBS15* contains 12 exons and a 3326 bp transcript is encoded by it. This gene encodes a planer cell polarity effectors protein fritz homologue with WD repeats (S. K. Kim *et al*., 2010).

1.3.16. Bardet-Biedl syndrome-16 (MIM 615993)

SDCCAG8 (serologically defined colon cancer antigen 8) is the responsible for BBS16 syndrome and its position is (1q43-q44). *BBS16* contains 18 exons and a 2632 bp transcript is encoded by it. The centrosome-associated protein encoded by *SDCCAG8* is localized at the distal extremities of both centrioles, and it co localizes with centrosomes throughout the cell cycle. Additionally, *SDCCAG8* was discovered in human retinal pigment epithelial cells, where it was found to be located close to centrosomes (Otto *et al*., 2010).

1.3.17. Bardet-Biedl syndrome-17 (MIM 615994)

LZTFL1 (leucine zipper transcription factor like 1) is the responsible for BBS17 syndrome and its position is (3p21.31). *BBS17* contains 10 exons and a 4073 bp transcript is encoded by it. Leucine zipper transcription factor-like protein 1 is encoded by it. It is a crucial negative regulator of the BBSome ciliary trafficking and the signaling of the sonic hedgehog pathway (Kiss *et al*., 2001; Marion *et al*., 2012; Seo *et al*., 2011).

1.3.18. Bardet-Biedl syndrome-18 (MIM 615995)

BBIP1 is the causative gene for BBS18 syndrome and its location is on chromosome 10 and its position on chromosome is (10q25.2). BBSome-interacting protein 1(BBIP1) is encoded by this gene. *BBIP1* contains 4 exons and a 2057 bp transcript is encoded by it. BBIP1 has main role in stabilization of cytoplasmic microtubules and ciliogenesis (Loktev *et al*., 2008; Scheidecker *et al*., 2014).

1.3.19. Bardet-Biedl syndrome-19 (MIM 615996)

IFT27 (Intraflagellar transport 27) is the causative gene for BBS19 syndrome and its location is on chromosome 22 and its position on chromosome is (22q12) consisting of 1020 bps. Two substantial (A and B) protein complexes, each having roughly 6 and 11 polypeptides, make up IFT particles. IFT27 stands out from the other IFT polypeptides examined because it is necessary for cell division and the continuation of the cell cycle (Bradley & Quarmby, 2005; Mahjoub *et al*., 2002; Qin, Wang, Diener, & Rosenbaum, 2007).

1.3.20. Bardet-Biedl syndrome-20 (OMIM 608040)

IFT74 gene is responsible for BBS20 syndrome and its location is on chromosome no chromosome 9 and its position on chromosome is (9p21.2) (Lindstrand *et al*., 2016). Rod-cone dystrophy, postaxial polydactyly, truncal obesity, renal anomalies, learning difficulty, hypogonadism in males, and genital abnormalities in females are all features of Bardet-Biedl syndrome-20 (BBS20), a rare autosomal recessive syndrome linked to ciliary dysfunction (Saida *et al*., 2014).

1.3.21. Bardet-Biedl syndrome-21

C8orf37 is the causative gene for BBS21 syndrome and its location is on chromosome no 8 and its position on chromosome is 8q22.1. BBS21 syndrome signs includes, RD, obesity, postaxial polydactyly and mild cognitive impairment (Heon *et al*., 2016).

1.10. Overview of BBS Proteins and their Interactions:

Numerous proteins associated to cilia are encoded by BBS genes and defects in these genes results in ciliopathy disorders (Tobin & Beales, 2009). The body of a vertebrate has both motile and nonmotile ciliated mammalian cell types that are widely distributed. Primary non motile cilia has 9+0 pattern with 9 microtubules triplets organized in a circle with an

outer membrane and these lack central microtubule pair and these are involved in left-right asymmetry, tissue formation, cell signaling and homeostasis (Satir, Pedersen, & Christensen, 2010). To produce and maintain cilia, which are crucial for cell movement, fluid transfer over epithelial cells, and sensory perception, intraflagellar transport (IFT) and an active transport of proteins along the microtubules in cilia are required. The BBS proteins are also involved in the development of cilia and the movement of certain proteins across cilia (Berbari *et al*., 2008; Jin *et al*., 2010).

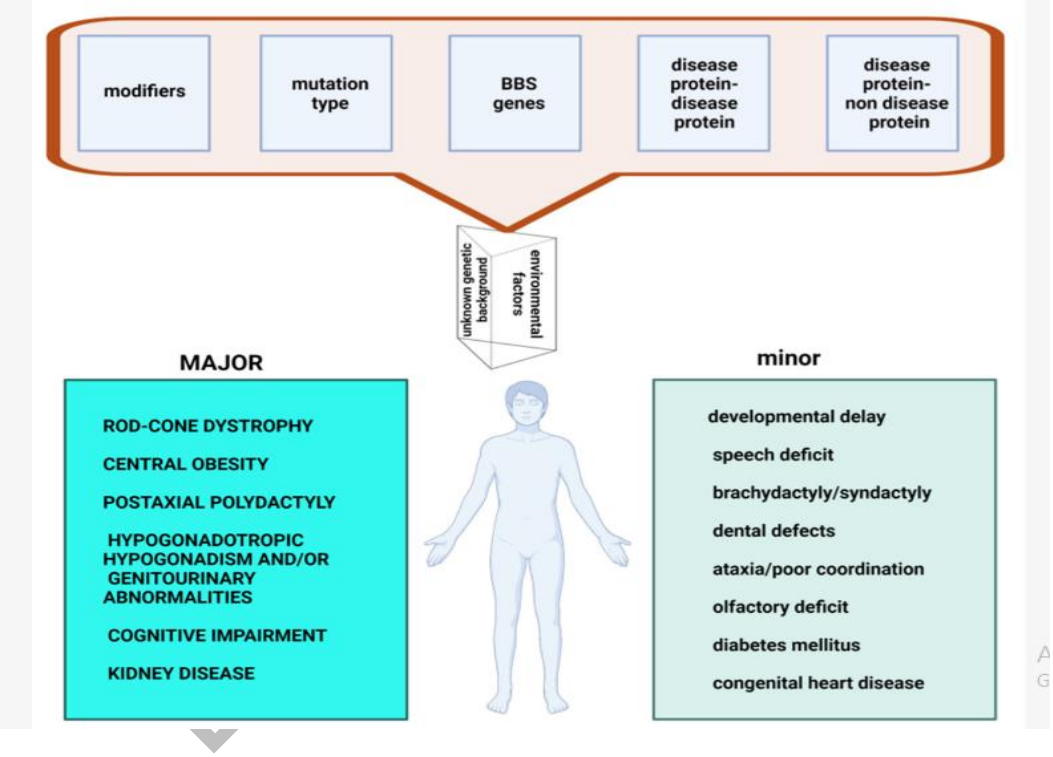

Fig.1.10: Different factors that result in BBS syndrome and the clinical signs of BBS syndrome. (Adopted from Florea, Caba *et al*, 2021)

BBSome complex is formed by the BBS[1,2,4,5,7,8,9 and 18] proteins and these proteins function in antero-grade and retro-grade transport at ciliary transition zone and the formation of this BBSome complex is promoted by the BBS-chaperonin complex formed by BBS6,BBS12,BBS10 with BBS7(Marion *et al*., 2012). For BBSome recruitment, the other BBS proteins operate independently in the centrosome or at the base of the cilium(Marion *et*

al., 2012). BBS3/ARL6 regulates the Wnt signaling and it also interacts with other BBS proteins and prevents the entry of ciliary vesicle into cilium. By producing a ring-shaped structure at the distal end of the transition fibres, ARL6/BBS3 facilitates or controls the change from vesicular to intraciliary trafficking(Rauch, 2011). E3 ubiquitin ligase is encoded by the BBS11 for BBS2 (Leitch *et al*., 2008). BBS [14, 15, 16] are involved in the recruitment of BBSome and these work at centriolar satellite while BBS17 has a negative effect on BBSome trafficking (Billingsley *et al*., 2010; Chiang *et al*., 2006; Sung & Leroux, 2013). BBS19 codes a part of the IFT-B complex that is necessary for the anterograde transport of ciliary proteins and is thought to be involved in connecting the BBS cargo to the IFT machinery (Chamling *et al*., 2014). BBS20 acts in conjugation with BBS4 and it is considered as a novel BBSome interacting protein and basically it is a centriolar satellite protein (Zhang, Yu, Seo, Stone, & Sheffield, 2012).

1.10.1. Disease Protein-Non Disease Protein Interaction

Keith *et al*. [2014] showed that there are numerous interconnectivity networks, and these functional interactions between complexes also explain the locus heterogeneity (Keith, Robertson, & Hentges, 2014; J. Lee & Chung, 2015; Mitchison & Valente, 2017). These interactions may be (Disease Protein-Non Disease Protein, Disease Protein-Disease Protein) interactions in case of disease protein-non disease protein interactions There is evidence for the existence of proteins that are connected to multiple proteins encoded by genes related to BBS syndrome. Aminoacylase-1 (ACY1) has relativity with BBS(2,4,7) gene products (Keith *et al*., 2014). The BBS proteins alone or in interaction with other proteins that are involved in locus heterogeneity can cause BBS syndrome or related disease(Keith *et al*., 2014).

1.10.2. Disease Protein-Disease Protein Interaction

BBSome consist of two parts head and body the head is formed by the BBS2 that interacts with BBS7 and the body is formed by proteins that are (BBS1,4,5,9 ,TTC8 and BBIP1) (Nager *et al*., 2017). BBIP1 is a central part of BBSome core and it interacts with TTC8 and BBS4 and thus plays an important role in assembly and stability of BBSome (Singh, Gui, Koh, Yip, & Brown, 2020). These interactions are necessary for a functioning BBSome and assist in explaining why patient disease is brought on by mutations at the BBS4-BBS1 and BBS8-BBS9 interactions (Klink, Gatsogiannis, Hofnagel, Wittinghofer, & Raunser, 2020).

1.10.3. Modifiers Role In BBS Syndrome

Modifier genes can change the phenotype of BBS syndrome. The PhenoModifier database contains 12 modifying variants in 6 genes that influence expressivity or pleiotropy (Sun *et al*., 2020). Therefore, this pleiotropic effect might be explained by the interaction between the two genes' products. Modifiers can change the BBS or MKS (Meckel-Gruber Syndrome) specific signs into non-specific signs (Leitch *et al*., 2008).

1.11. Localization of BBS Proteins and their Function

Almost 26 genes associated with BBS syndrome has been discovered until now. Most of the BBS proteins coded by these genes localize to base of cilium and have role in ciliary functioning and biogenesis(RL Forsyth & Syndrome, 1993). BBSome is a macromolecular complex formed by (BBS1,2,4,5,7,9,BBS8/TTC8 and BBS18/BBIP1) proteins and it works as an adopter for those molecules that are involved in intraflagellar transport (Jin *et al*., 2010; Nachury *et al*., 2007). Molecules that are involved in intraflagellar transport undergoes bidirectional movement along with microtubule backbone and thus gave rise to IFT-A and

IFT-B protein complex and act as carrier for proteins involved in ciliary homeostasis or signaling (Lechtreck, 2015). IFT-B complex is involved in anterograde IFT and have IFT172, IFT74/BBS20 and IFT27/BBS19 components (Bhogaraju, Engel, & Lorentzen, 2013). Ciliary export of hedgehog signaling molecule is modulated by the interaction of BBS19/IFT27 with ADP ribosylation factor like GTPase 6 (ARL6)/BBS3. It is also proposed that BBS19/IFT27 interface with BBSome through lucine zipper transcription factor like 1 (LZTFL1)/BBS17 (Eguether *et al*., 2014; Liew *et al*., 2014). An interaction of BBS19/IFT27 and BBS20/IFT74 has been found.IFT172 performs a major role in the stability of cilium (Brown, Cochran, Craige, Kubo, & Witman, 2015; Bujakowska *et al*., 2015). BBS3/ARL6 is a GTPase that is involved in the recruitment of BBSome to ciliary membrane and in this way it stabilizes the BBSome position within the cilium(Jin *et al*., 2010).

Chaperonin complex involved in BBSome assembly consist of BBS12,BBS10 and MKKS/BBS6 (Álvarez-Satta, Castro-Sánchez, & Valverde, 2017; Seo *et al*., 2010). MKS1/BBS13, CEP290/BBS14, WDPCP/BBS15, and SDCCAG8/BBS16 are among the proteins that work at the basal body and are engaged in ciliogenesis and the regulation of trafficking of BBSome within the ciliary compartment (Barbelanne, Hossain, Chan, Peränen, & Tsang, 2015; Dawe *et al*., 2007; Williams *et al*., 2011). It has been demonstrated that nephrocystin 1 (NPHP1), which is located in ciliary transition zone, controls the early stages of cilia formation (Lindstrand *et al*., 2014; Williams *et al*., 2011). It is hypothesized that BBSome activity is also regulated by the interaction of BBS9 with LZTFL1/BBS17 while E3 ubiquitin ligase that is tripartite motif containing 32/BBS11 controls cytoskeleton components (Das, Qian, & Tsang, 2017; Marion *et al*., 2012; Seo *et al*., 2011). A protein known as cilia and flagella associated protein (CFAP)418/BBS21, which is found at the base of the cilium, appears to play a part in promoting protein transport, still its exact purpose and mechanism of association within the other BBS proteins is unclear (Heon *et al*., 2016).

Centrosomal protein (CEP)164 and Sodium channel and clathrin linker 1 (SCLT1) are compulsory for ciliary initiation and have recently been associated with BBS and these are parts of distal appendages that are accountable for docking the cilium to the plasma membrane (Morisada *et al*., 2020; Shamseldin *et al*., 2022; Yang *et al*., 2018). S-phase cyclin A associated protein in ER (SCAPER) is recently shown to be associated with BBS and it is involved in ciliary tip localization and this role suggests its involvement in ciliary dynamics during cell cycle (Wormser *et al*., 2019).

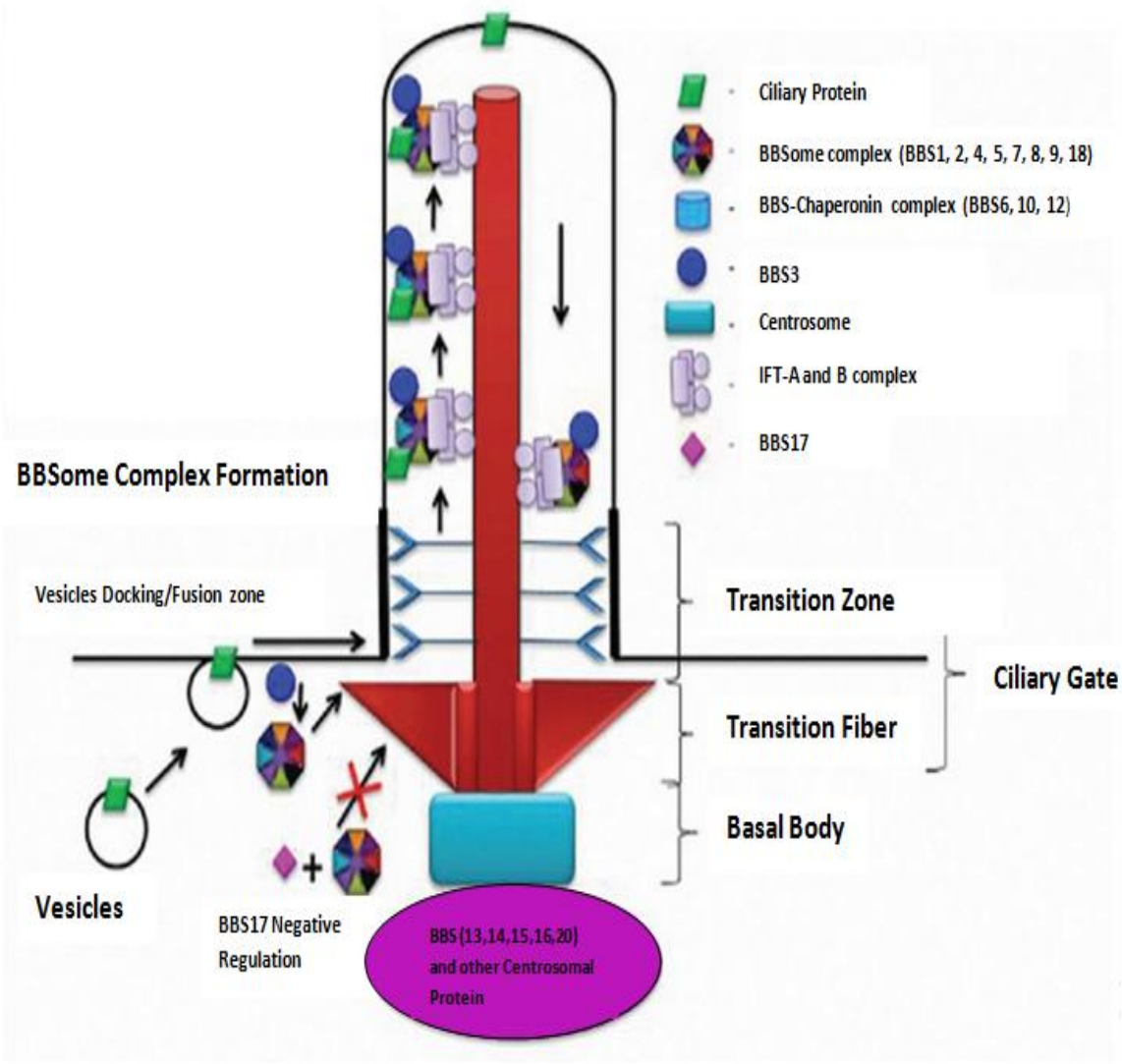

Fig.1.11: Localization of BBS proteins. (Adopted from Priya, Nampoothiri *et al*, 2016)

1.12. Mutational Heterogeneity of BBS Genes

A study conducted by Florea, Caba *et al* [2021] by assessing Human Gene Mutation Database Professional (HGMD®)2021.1 database showed different types of 647 pathogenic genetic modifications in genes that are implicated in BBS. Genes that are involved in BBSome formation possess different type of mutations such as small indels, small insertion/duplications, gross deletions, small deletions, complex rearrangement, splicing substitutions, gross insertion/deletions and nonsense/missense mutations. Other genes implicated in BBS possess these mutations such as nonsense/missense, small insertion/duplications, and small deletions. Splicing variants, copy number variants, nonsense and frameshift mutations may result in loss-of-function (LOF) mutations that causes some forms of BBS syndrome(Florea, Caba, & Gorduza, 2021). In figure 1.12 pathogenic variants are shown in those genes that are involved in in BBSome such as *(BBS1,BBS 2, BBS4, BBS5, BBS7, TTC8 ,BBS9)* and in BBSome interacting protein 1 *(BBIP1).*

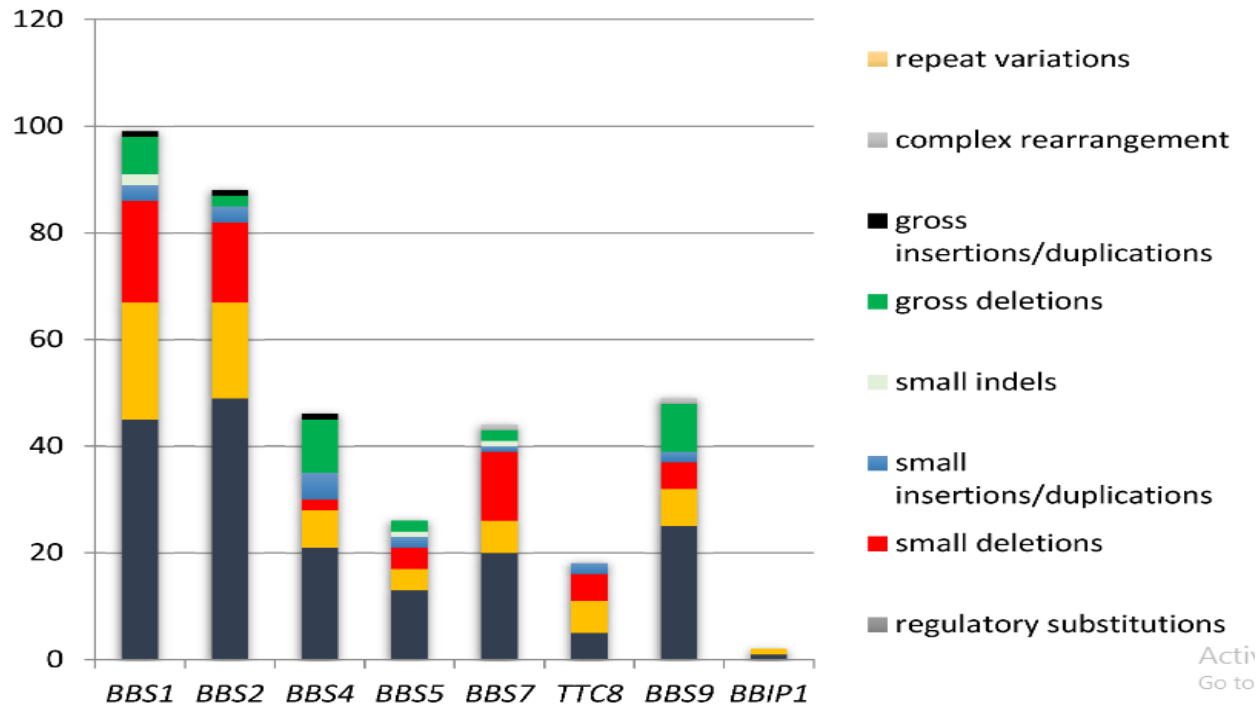

Fig.1.12: Pathogenic variants in BBSome. (Adopted from Florea, Caba *et al*, 2021)

In figure 1.13 pathogenic variants in other genes such as (*ARL6, MKKS, BBS10, TRIM32, BBS12, MKS1, CEP290, WDPCP, SDCCAG8, LZTFL1, IFT27, IFT74, C8orf37, SCLT1, NPHP1*, and *SCAPER*) are shown.

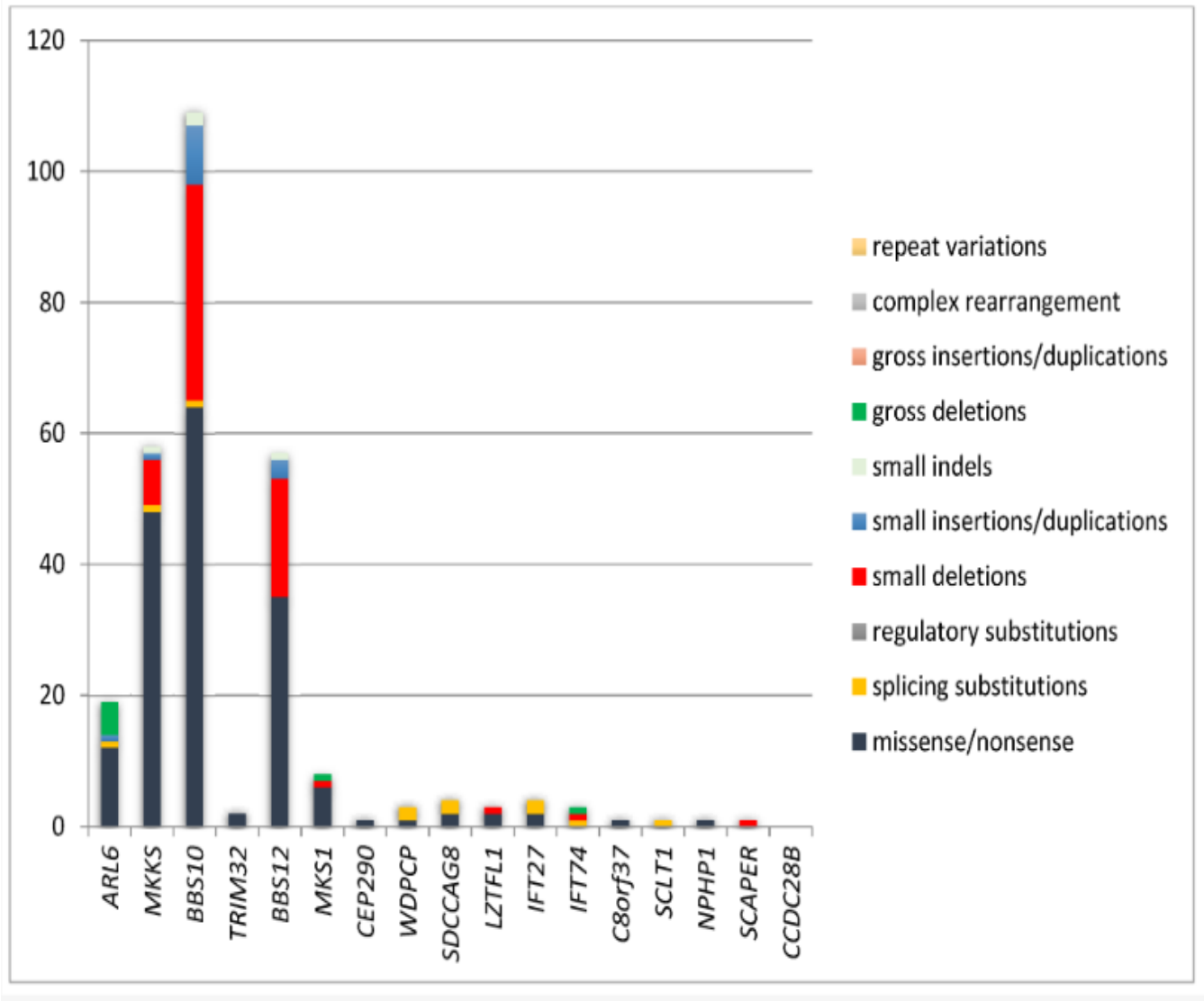

Fig.1.13: Pathogenic variants in genes that are involved in BBS syndrome. (Adopted from Florea, Caba *et al*, 2021)

1.6.1 Mutation Spectrum of BBS genes in Pakistan

The prevalence frequency of BBS is yet not reported in Pakistan. Until now limited amount of study is conducted on BBS in Pakistan. There are certain reasons behind the limited study of BBS but the main reason is the lack of proper diagnosis of BBS syndrome and its low prevalence. No mutation hotspot is predicted in Pakistan until now. In India ARL6 and BBS10 are most mutated genes in case of BBS syndrome (Chandrasekar, Namboothiri, Sen, & Sarangapani, 2018). BBS10 is considered as mutational hotspot in India in case of BBS syndrome (Chandrasekar *et al*., 2018; Pasinska, Dudarewicz, Jakubowski, & Haus, 2015). In table 1.3 different reported BBS genes mutations in Pakistan are listed.

Table 1.3: Mutation Profile of BBS genes in Pakistan.

Sr.No	Gene	cDNA Variant	Protein Variant	Region	References
1	*BBS1*	c.47+1G>T		Punjab	(Ajmal *et al*., 2013)
2	*BBS1*	c.442G>A	p.(Asp148Asn)	Punjab	(Ajmal *et al*., 2013)
3	*BBS2*	c.443A>T	p.N148I	Kashmir	(Ali *et al*., 2021)
4	*BBS2*	c.1658C>T	p.R413X		(Chen *et al*., 2011)
5	*ARL6*	c.123+1118del53985†			(Chen *et al*., 2011)
6	*ARL6*	c.281T>C	p.Ile94Thr		(Saadullah Khan *et al*., 2013)
7	*ARL6*	c.534A > G	p.(Q178Q)		(Maria *et al*., 2016)

8	*BBS5*	c.734_744del	p.(E245Gfs*18)		(Arts & Qamar)
9	*BBS6*	c.119C>G	(p.Ser40*)	Sindh	(Ullah *et al*., 2017)
10	*BBS6*	c.775-775delA			(Ullah *et al*., 2018)
11	*BBS6*	c.287C>T			(Ullah *et al*., 2018)
12	*BBS7*	c.719G>T	p. Gly240Val		(Hayat *et al*., 2020)
13	*BBS7*	c.580_582delGCA		KPK	(Ullah *et al*., 2017)
14	*BBS7*	c.1677–1 G > A	p. (Arg559ArgfsTer3)		(Shaukat, Ishaq, Muhammad, & Naz, 2020)
15	*BBS7*	c.1592_1597delTTCC AG		AJK	(Ullah *et al*., 2017)
16	*BBS8*	c.1347G>C	p.Gln449His	KPK	(Ullah *et al*., 2017)
17	*BBS9*	c.702 + 1del.			(Suárez-González, Seidel, Andrés-Zayas, Izquierdo, & Buño, 2021)
18	*BBS9*	c.299delC	(p.Ser100Leufs*24)		(Muzammal *et al*., 2019)
19	*BBS9*	c.1131G>A		AJK	(Ain-ul-Batool, Blasius, Kaindl, & Ghazanfar, 2019)
20	*BBS9*		p.R75X		(Niazi *et al*., 2019)
21	*BBS9*		p.R481X		(Niazi *et al*., 2019)
22	*BBS10*	c.1958_1967del			(Agha *et al*., 2013)
23	*BBS10*	c.271_272insT		AJK	(Ullah *et al*., 2017)
24	*BBS10*	c.1075C>T	p.Gln359*		(Saadullah Khan *et al*., 2013)
25	*BBS12*	c.2014G > A	p.(A672T)		(Maria *et al*., 2016)
26	*BBS12*		p.S701X		(Pawlik *et al*., 2010)
27	*CEP164*	c.277C > T	p.(R93W)		(Maria *et al*., 2016)

1.7 Techniques used to identify the genetic basis of BBS

Now a day's different mapping techniques are used to identify mutated genes in patients with inherited diseases and technique selection depends on disease phenotype and pedigree structure. When pattern of inheritance and phenotype is clear then the selection of suitable gene is done.

1.7.1 Sanger sequencing of gene

Sanger sequencing is a gold standard and it is used to analyze the mutations in DNA. This method is used to examine the genetic changes associated with disorders for which the causal gene is known. The foundation of sanger sequencing is the inclusion of dideoxynucleotides during single-stranded DNA molecule copying, which causes chain termination at random and produces fragments of various sizes. These fluorescently marked pieces are then electrophoretically separated, with a laser scanner inducing the fluorescence and a diode array detecting it(Sanger & Coulson, 1975). In present study we have used this technique to find DNA changes in the selected region.

1.7.2 Linkage Analysis

Linkage analysis is performed for those clinical phenotypes in which involvement of specific gene is not clearly mentioned. For BBS linkage analysis is the best technique. In linkage analysis the disease associated loci and genes are mapped. The genome regions that are the same in affected family members but different in unaffected relatives can be identified via linkage analysis. Examination of recombination events between two distinct loci is a key component in establishing genetic linkage. Additionally, the availability of appropriate

genetic markers is a requirement for linkage analysis (Strachan & Read, 2011; Teare & Barrett, 2005).

1.7.3 Genotyping using microsatellite markers

Microsatellite markers are short, di-, tri-, and tetra-nucleotide repeats found on several chromosomes that are highly polymorphic DNA sequences. These microsatellite markers are frequently employed to map many disease-causing genes in the human genome as these have widespread distribution in the genome. In this approach of homozygosity mapping, the individuals are genotyped using microsatellite markers to identify the homozygous DNA segments on various chromosomes, referred to as haplotype blocks that are shared by every member of the affected family. To identify the candidate genes that contain the disease-causing mutations, additional research is conducted on these shared areas. By computing the logarithm of the odds (LOD) score using genotyped haplotype blocks in both affected and healthy people, significance of linkage is investigated(Morton, 1955).

1.7.4 Genotyping single nucleotide polymorphisms

Each normal human contains 23 chromosome pairs consisting of almost 25000 genes, encoding proteins. There is 99.5% genome similarity among unrelated individuals the remaining difference is due to copy number variants (CNVs that are insertions, deletions) ,inversions and single nucleotide polymorphism(SNPs). Almost 3.2 million SNPs are present in coding and non coding sequence of DNA of an individual and may be synonymous or nonsynonymous and mostly these do not hinder any functional effect. About 10400 nonsynonymous SNPs are detected in human exome out of which only 1500 are predicted to affect protein function. Due to their widespread distribution throughout the genome, SNPs

have the potential to be an effective tool for identifying homozygous areas in linkage research. A recently created method for homozygosity mapping is high-density SNP microarray analysis. It is better than microsatellite markers (Gunderson, Steemers, Lee, Mendoza, & Chee, 2005; Levy *et al*., 2007; Ng *et al*., 2008).

1.7.5 Exome Sequencing

Exome sequencing can be used to identify the genetic basis of different inherited diseases. Next generation sequencing (NGS) is most popular technique now-a-days and it is used for diagnostic and research purposes. This technique is used for the identification of disease associated genes and this technique is highly cost effective and robustic. Several different NGS platforms are now being used for enriching and sequencing operations. Now a day's genetic basis of most of the diseases are assessed through exome sequencing (Majewski, Schwartzentruber, Lalonde, Montpetit, & Jabado, 2011; Metzker, 2010).

1.8 Current Management of BBS Syndrome and Future Therapies of BBS Syndrome

In BBS patients different symptoms are observed such as hypertension, diabetes and metabolic disorders. In recent treatment approach the primary focus is on management of these symptoms so that the organ systems previously affected by BBS particularly eyes and kidneys should be prevented from secondary impact of these conditions. For most patients the weight control is primary focus and some of them prefer to have bariatric surgery while other prefer to have anti obesity medication but diet input is most effective method of weight loss for most of the patients. In UK those patients who visit BBS clinics are advised to attend

multidisciplinary clinics so that they can be properly monitored for severity of different symptoms and on diagnosis basis they should be assigned medications.

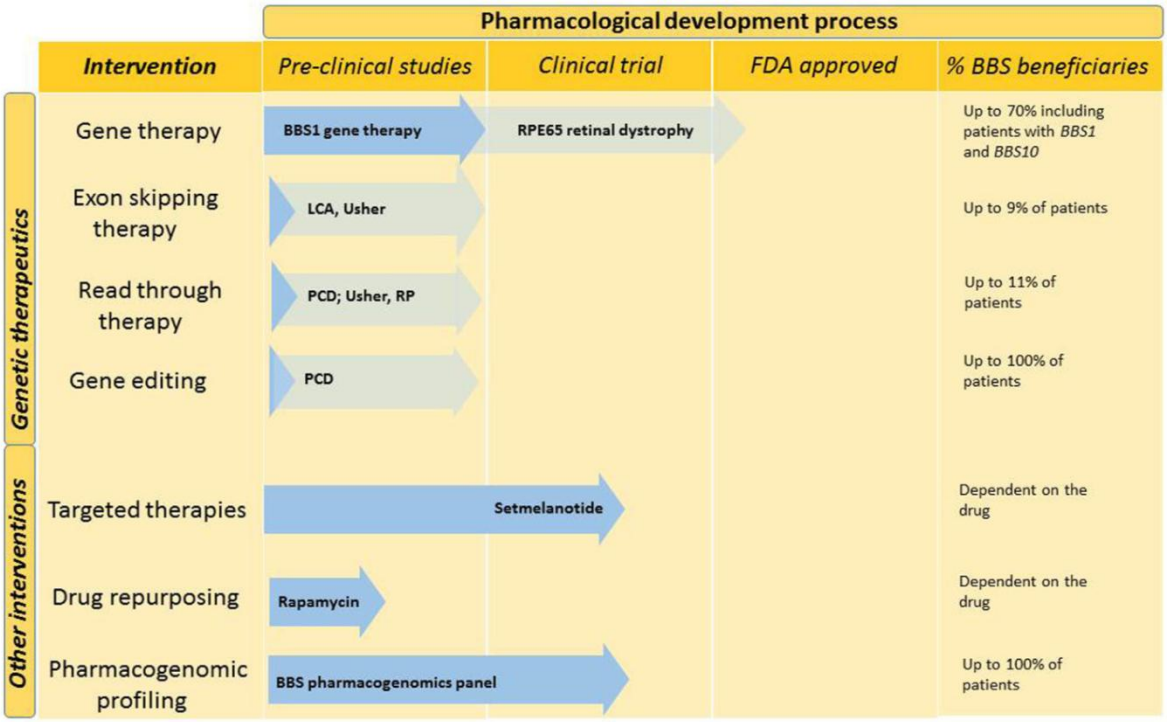

Fig.1.14: Phase of the pharmaceutical development process and upcoming initiatives. PCD, primary ciliary dyskinesia; LCA, leber congenital amaurosis; US, usher syndrome. (Adopted from Forsythe, Kenny *et al*. 2018)

1.8.1 Gene Therapy

Gene therapy has high success rate and different gene therapies has been developed and main focus is on development of gene therapy for rod-cone dystrophy. Viral and non-viral vectors are used for gene therapy but the main hurdle in the development of gene therapy is the development of long lasting, safe and effective gene therapy. A successful retinal gene therapy has been developed for RPE65 associated LCA and work in ongoing for the development of retinal gene therapy of BBS (Bainbridge *et al*., 2015; Dias *et al*., 2018; H. Lee & Lotery, 2017).

1.8.2 Read Through Therapy

Nonsense mutations are responsible for premature protein termination and results in the formation of incomplete defective proteins that are laterally degraded. Read-through therapy is involved in destabilizing the ribosome translational response to nonsense mutation and allowing insertion of amino-acyl-tRNA and hence formation of full length protein occurs that plays role in minimizing the severity of disease. Read-through therapy is under clinical and preclinical trials for cystic fibrosis, Duchene muscular dystrophy and many ciliopathies such as usher syndrome, RP and ciliary dyskinesia (Bukowy-Bieryllo, Dabrowski, Witt, & Zietkiewicz, 2016; Finkel *et al*., 2013; Goldmann, Overlack, Wolfrum, & Nagel-Wolfrum, 2011; H.-L. R. Lee & Dougherty, 2012; Mutyam *et al*., 2016; Mutyam *et al*., 2017; Oren, Pranke, Kerem, & Sermet-Gaudelus, 2017; Reinig, Mirzaei, & Berlau, 2017; Richardson, Smart, Tracey-White, Webster, & Moosajee, 2017; Schwarz *et al*., 2015; Xue *et al*., 2017).

1.8.3 Exon Skipping Therapy

Exon skipping therapy is performed by using antisense oligonucleotides to skip exon containing undesirable sequence and as a result a novel splicing product is produced that may perform wild type function to a greater extent and this therapy is performed for those mutations that results in complete loss of function. Exon skipping therapy is under clinical trials for spinal muscular atrophy and Duchenne muscular dystrophy and it is under preclinical trials for ciliopathies such as LCA and Usher syndrome. Only 9% patients can

utilize this therapy but challenge is that mutations in BBS are private and require individual therapy (Garanto *et al*., 2016; Garanto & Collin, 2018; Goemans *et al*., 2011; Miller *et al*., 2013).

1.8.4 Genome Editing

Genome editing allows DNA deletion, correction and replacement and this process is done by using targeted endonuclease that creates double-stranded breaks in DNA at specific position then DNA repair mechanism repairs these gaps and wild type is restored. Successful genome editing is performed on the embryo at risk for hypertrophic cardiomyopathy caused by mutation in *MYBPC3* and for treatment-resistance leukemia. Safety, specifity and off-target DNA damage are the main challenges in this technique (Lai *et al*., 2016; Lux & Scharenberg, 2017; Ma *et al*., 2017; Wu *et al*., 2017; Zheng, Li, & Tsang, 2015).

1.8.5 Targeted Therapies

Work on targeted drug therapies for BBS is ongoing and it will be a novel development. Recently melanocortin receptor agonists are potential therauptic agent against obesity in BBS (Seo *et al*., 2009).

1.9. Objectives:

- To study demographic and clinical features of BBS patients.
- To screen exon 2 and exon 5 of *ARL6* gene of selected BBS families for mutation detection.

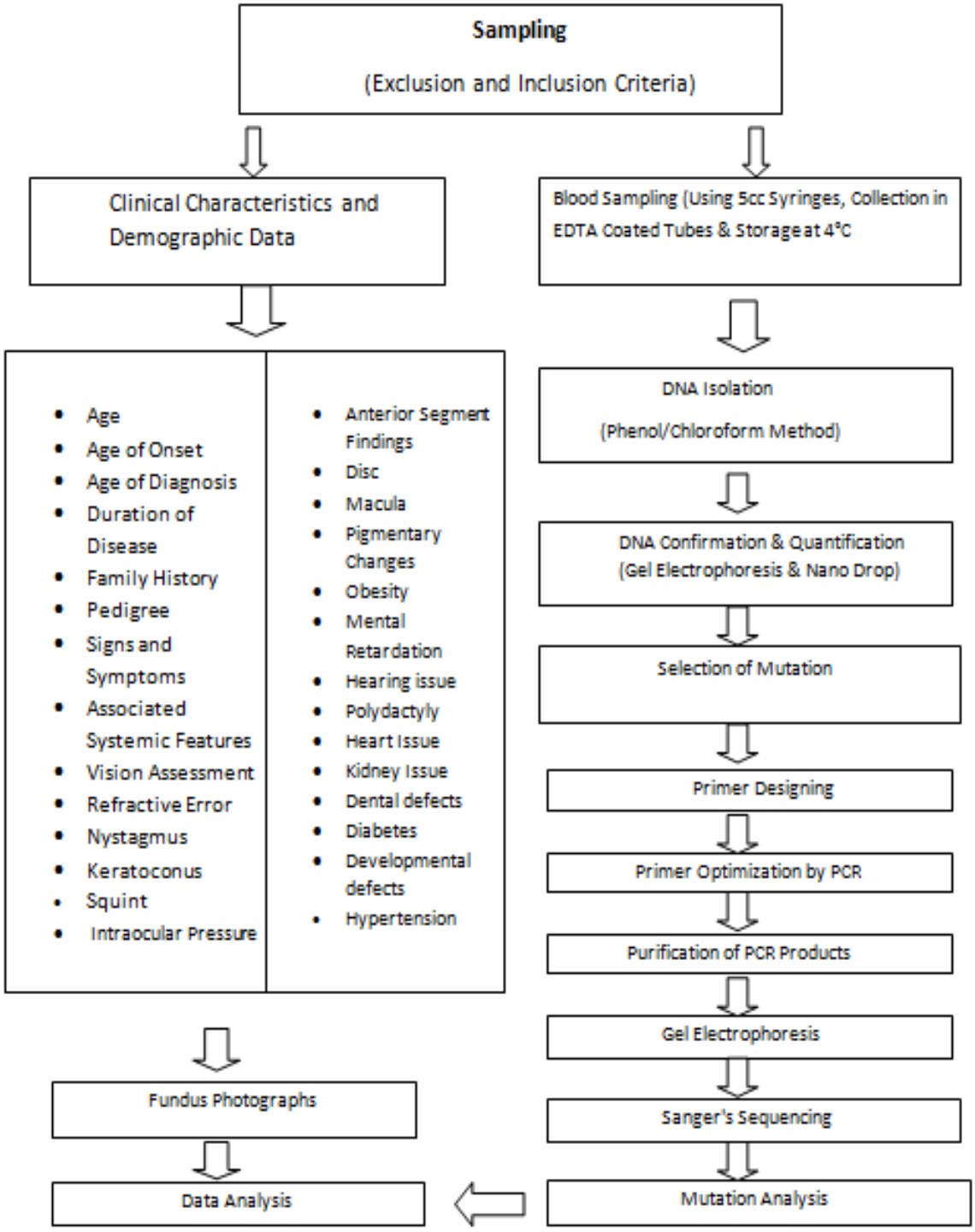

Fig.2.1: Steps of Methodology

Chapter No 2:

MATERIALS AND METHODS

2.1. Ethical Approval:

The present study was conducted under the Department of Zoology Quaid-i-Azam University Islamabad and its consent was attained by the Bioethical review committee of Quaid-i-Azam University and Al-Shifa Eye Trust Hospital Rawalpindi.

2.2. Recruitment of Families:

Bardet-Biedl Syndrome Patients were separated after conducting a long detailed interview from those patients that were clinically diagnosed with Retinitis Pigmentosa (RP). BBS patients and their close relatives from different parts of Pakistan were included in this study.

2.3. Pedigrees of Families:

A thorough interview with the typical family leader was done in order to ascertain the genetic link between the family members. The pedigree analysis revealed the mode of inheritance. Each family's pedigree was depicted using HaploPainter 1.043. The circle represents the female in the sketched pedigrees, whereas the square represents the male. Patients with Bardet-Biedl syndrome are represented by the filled symbols, whereas healthy people are represented by the hollow symbols. The departed people are represented by the diagonal line on the symbol. The cousin marriage is shown by the two double lines between the two symbols. Roman numbers were employed to indicate the several generations, while Arabic numerals were utilized to identify the specific members of each generation.

2.4. Criteria of Patient Selection for Blood Collection:

At the Al-Shifa Eye Trust Hospital in Rawalpindi, the blood samples of the affected families from various parts of Pakistan were taken. All of the study participants agreed to participate in sampling, and their doctors made a clinical Retinitus Pigmentosa (RP) diagnosis for them in accordance with WHO guidelines and after that BBS patients were extracted by conducting a detailed interview of patients.

2.5. Families Inclusion and Exclusion Criteria:

Those families having syndromic RP were included in the study but only those patients that were possessing BBS syndrome features were analyzed for molecular analysis. Patients having eye disorders other than RP, non-syndromic RP or RP in syndromic condition other than BBS were not included in the study. Those patients that were fulfilling inclusion criteria but were not willing to participate in the study were also excluded.

2.6. Collection of Samples:

All participants gave written consent before having their 5 ml of blood drawn into appropriately labelled EDTA vacutainer tubes. The label contained the person's name, parent's name, and the unique anonymous identification (LAI) number that was given to each person in accordance with how to keep them distinct from one another. For avoiding blood clotting EDTA containing tubes were well shacked after filling with blood and then stored at -20°C in a Dawlance refrigerator.

2.7. Work Done at Molecular Biology Lab Quaid-i-Azam University:

- **Solution preparation for DNA extraction.**
- **Extraction of DNA**
- **Electrophoresis by using Agarose Gel**
- **Nano drop**
- **Primer designing**
- **Primer dilutions**
- **Polymerase Chain Reaction (PCR)**
- **PCR product purification**
- **Sequencing**

2.7.1. PREPARATION OF REAGENTS FOR DNA EXTRACTION

- **Solution A**

Solution A is known as lysis solution and it consist of 0.32 M sucrose, 10mM Tris (pH-7.5), 5mM MgCl and Triton X 100 (1% V/V).

- **Solution B**

Solution B is made up of 400mM NaCl, 10mM Tris (pH 7.5), and 2mM EDTA (pH 8.0).

- **Solution C**

In DNA extraction, solution C is known as phenol, which aids in the removal of lipids and non-polar proteins from the solution.

- **Solution D**

- Chloroform and isoamyl alcohol make up solution D in a 24:1 ratio. Chloroform is used to eliminate phenol from DNA. Additionally, it aids in protein removal. Foaming that develops after DNA extraction using the phenol chloroform technique is eliminated by isoamyl alcohol. Changes in the chloroform/isoamyl alcohol ratio cause the extraction of additional sample components, including rRNA.

- **Proteinase K**

100mg/ml working concentration was used.

- **20%SDS**

10 g Sodium dodecyl Sulphate in 50 mL water.

- **70% Ethanol**

60ml distilled water was added 140ml absolute ethanol (molecular grade) to prepare 70% ethanol.

- **0.2 mM TE Buffer**

1 mM EDTA, 10 mM Tris hydroxyl (methylamino) methane, (pH 8.0).

2.7.2. DNA EXTRACTION

The Phenol-Chloroform Method, an organic extraction technique, was utilized to extract the genomic DNA. The procedure for isolating genomic DNA is described below.

A. FIRST DAY:

- For the red blood cell (RBC) lysis, blood samples were defrosted and allowed to thaw at room temperature.

- Eppendorf of 1.5ml capacity were taken and then labeled with persons UAI and then blood sample of approximately 600ul of labeled person and 600µl of Solution A were injected in this tube through pipette.
- Then this blood and Solution A mixture was well shacked for thorough mixing and then left for 30 minutes for appropriate lysis.
- After 30 minutes lysis centrifugation was done in Beckman microfuge at 13000rpm for 10 minutes.
- After centrifugation half of supernatant was discarded in an appropriate beaker containing water mixed with bleach for avoiding any contamination.
- The remaining quantity containing pellet was again mixed with 400µl Solution A and was well shacked for thorough mixing and then again centrifuged at13000rpm for 10 minutes.
- After centrifugation almost all the supernatant was discarded and then pellet was dissolved with 400ul Solution A and then centrifuged at 13000rpm for 10 minutes.
- After centrifugation all the supernatant was discarded and pellet was dissolved in 400ul Solution B, 6ul Proteinase k and 25µl of 20%SDS by vigorous shaking.
- These sample containing eppendorf tubes were incubated at 37°C for overnight for protein digestion in WBCs pellet.

B. Second Day:

- The second day, a new combination of solutions C and D was made by combining both solutions equally (50:50). Centrifugation of each sample was done for 10 minutes at 13,000 rpm after adding 500ul of freshly made solution C plus D.
- There were created two layers (top and bottom) as a result of centrifugation. The top layer was removed and moved to an eppendorf with new labels.

- Add 500µl of solution D to the separated layer, shake erratically, and centrifuge at 13000 rpm for 10 min. There were two layers created. The sample's top layer was removed and transferred to fresh, labeled Eppendorf tubes. Next, 55 ml of chilled sodium acetate (3M, pH 5-6) and 500ml of chilled iso-propanol were added to the sample. To precipitate genomic DNA, the sample was flipped over several times.
- After that, the sample was centrifuged for 10 minutes at 13,000 rpm, and a pellet was produced as a result.
- After discarding the supernatant, the pellet was rinsed with 200µl of 70% ethanol and centrifuged for 10 minutes at 13,000 rpm.
- The Supernatant was once more discarded, and the pellet was left to dry in the vacuum concentrator.
- A appropriate volume (80-200µl) of TE buffer (Tris-EDTA) was added to an eppendorf tube with air dried DNA, and the tube was then incubated overnight at 37°C.

C. Third Day:

- Parafilm strips were used for sealing the eppendorf tubes caps.
- After sealing, samples were subjected to a 1-hour heat shock in a water bath at 73 °C to prevent denaturation of the extracted DNA.
- Tubes were placed at room temperature for 5 minutes and then DNA solutions were centrifuged and transferred in labeled autoclaved screw cap tubes.
- Then 4µl of the extracted genomic DNA from each sample was combined with 4µl of bromophenol blue dye and run on a 1% agarose gel for qualitative and quantitative analysis. The results were then analysed using a UV transilluminator (Gel-Doc system) from (Biometra, Gottingen, Germany) and stored at -20 °C in a cryobox.

2.7.3. QUANTITY AND PURITY ASSESSMENT OF DNA:

Two different methods were used for determining the concentrations of DNA.

A. Agarose Gel Electrophoresis (1%):

- Gel electrophoresis was used for the validation of the DNA sample that had been extracted. Below is a description of the Agarose Gel Electrophoresis (1%) methodology.
- In a conical flask, 0.5 grammes of agarose powder was dissolved in 50 ml of IX TBE (Tris-Boric Acid-EDTA) buffer to create a 1% agarose gel.
- In a 1000 ml bottle, 100 ml of 10X TBE was combined with 900 ml of distilled water to create a 1X TBE buffer. The 10X TBE buffer was created by mixing 40 ml of 0.5M EDTA, 108 g of Tris, 54 g of Boric acid, and then 1000 ml of final volume was obtained by adding distilled water into the mixture. The pH was then set at 8.0.
- The mixture was microwaved (Dawlance) for 1-2 minutes to get a clear solution. The opening of the flask was covered with aluminum foil before being placed in the microwave.
- After that, it is left to cool for a few minutes at room temperature.
- 5µl of Ethidium Bromide (EtBr) was added to the gel mixture. Under ultraviolet (UV) light, ER, an intercalating agent, is utilized to identify DNA.
- Comb and casting tray were set up in a rack.
- The casting tray was filled with the clear solution, making sure that no bubbles formed there and then allowed it to polymerize (solidify) at room temperature for 30–40 minutes.
- The comb was carefully removed when the gel had solidified, and the gel was then placed in the gel tank (Cleaver Scientific Limited CS-3000V), that was filled with the IX TBE buffer, a running buffer.

- 3µl of each sample's extracted DNA and 3µl of 6X Bromophenol blue(Loading dye) were combined to create the loading samples.
- For 25 minutes, the gel electrophoresis device was operated at 120 volts.
- Using the Gel Documentation System (Cleaver Scientific Limited), the gel was seen under UV once running was finished.
- The image was saved on computer for future use.

Table 2.1: Composition of agarose gel and other required chemicals.

S.No	Solutions	Compositions of Different Required Solutions
1	**Agarose Gel 1% In 50ml**	0.5g Agarose + remaning volume upto 50ml filled with 1X TBE buffer + 2ul Ethidium Bromide
2	**Agarose Gel 2% In 50ml**	1g Agarose + remaning volume upto 50ml filled with 1X TBE buffer + 5ul Ethidium Bromide
3	**10X TBE Buffer used as Gel Preparation Buffer**	EDTA(3.65g)+Tris(54g)+Boric Acid(27.5g)+Deionized water upto 500ml
4	**Ethidum Bromide(100ml)**	Ethidium Bromide (1g)+ Auto claved filtered water upto 100ml
5	**1X TBE Buffer 1000ml (Gel Running Buffer)**	900ml distelled water + 100ml 10X TBE
6	**Bromophenol (Loading Dye 25ml)**	Auto claved filtered water upto 25ml Sucrose 10g + Bromo-phenol blue(0.0875)

B. Nanodrop:

Using TE buffer as a blank, the DNA samples were quantified (concentration) and qualified (purity) on nanodrop (Thermo-Scientific 2000) to determine the concentration and purity of the extracted DNA.

Table 2.2: DNA quantity, purity and absorbance of Bardet-Biedl syndrome patients belonging to selected families for molecular analysis.

Sr. No	Family ID (LAB ID)	Patient ID Proband	OD AT 260 A°	OD AT 280 A°	RATIO	Conc.of Stock (ng/ul)
1	RP 04	V-1	3.90	2.07	1.88	180.82
2	RP 06	III-5	2.85	1.42	2.00	148.75
3	RP 21	IV-1	2.95	1.52	1.95	147.75
4	RP 23	III-1	2.22	1.60	1.38	156.02
5	RP 43	IV-4	1.86	1.63	1.14	108.32
6	RP 46	V-9	3.42	1.89	1.80	136.36

2.7.4. Primer Designing For Selected Region Amplification:

Primer3 software (http://bioinfo.ut.ee/primer30) was used to create primers for the amplification of the BBS3/ARL6 gene's designated DNA region (exon 2 and 5). Conditions such the primer's annealing temperature, the salt concentration, the size of the amplicon it could amplify, and the length of the primer needed were adjusted to the optimum level. The ensemble website (https://asia ensembl.org) provided the reference sequence that was used to create the primer. The specificity of the chosen primers was verified using the Blast like alignment tool (BLAT) on the UCSC genome browser (https://genome.ucsc.edu/cgi-bin/hgBlat). Additionally, the In-Silico PCR tool (https://genome.ucsc.edu/cgibin/hgPer?hgsid6560538798cQDaXBwie7X3YmOGd5sY7jwxf41) on the UCSC genome browser was used to approve the amplicon size for the primer pair. The Table lists the order of primers, melting, and product size.

Table 2.3: Primers for exon 2 and 5 of *ARL6* gene.

GENE	EXON	FORWARD PRIMER (5´ ⟶3´)	REVERSE PRIMER(5´⟶3´)	PRODUCT SIZE (bp)	TM °C
BBS3/ARL6	2	GCTCCTTTCTGGTAATCAGCTT (Length 22 bp)	TGCAAATTATGGCTGAGGAT (Length 20 bp)	478	59
BBS3/ARL6	5	TTGCATCGGTGTAATAGGGTTA (Length 22 bp)	AGACACCCCAAAAACAATGC (Length 20 bp)	653	59.4

2.7.4.1. FASTA Sequence of Selected Exon 2 of *ARL6*:

TTAAATATGATAAATTACAGAGAGAAATGTTGTTATTTCCAAGGCATTGTTCTGTGTT
GCCACCTGTTATAAGCTGCAAATCTGCGTGTCAGTTAAGGCTCCTTTCTGGTAATCAG
CTTACACTGTGTTTACTAAGTGCAAAGCTACATTGACATAGTTTTCACACTATTATTA
TGTGTATTTAAATTATTACCTTTTTTTAATACACCTACCAATATTTTCCATAACTTAA
GGTGCCTTTGGGTAATATTTTATTTTTTCTTAATTGCAGCTGGTTTGTAAATATTTGA
ATCACATTATGGGATTGCTAGACAGACTTTCAGTCTTGCTTGGCCTGAAGAAGAAGGA
GGTTCATGTTTTGTGCCTTGGGCTAGATAATAGTGGCAAAACGACGATCATTAACAAA
CTTAAACCTTCAAATGTAAGTATCTTTGTTAGATGCTTTATGTATTTTCTGCTACTAA
AGAAAATTAATGTGCAGAATTATGTTATATGACGTTAAAACCGCATATAATCACATTA
AGATATTCTGTTAACCTTTCAGTACCTTTAAGTATCCTCAGCCATAATTTGCAGTATA
TTTTACACAGTAAATTTTAATTATTAAACTGGAATATTATTTTCAAATGTCTTTTCTT
TCAAAAGTTCTTCACAGATTTACTGAGTTTACTTTAAATGTCATTGCCTTCTTTTTAG
TTTCCTCCTTACAGGAGGAGCAAAACAGAGAAAGGTGAAACATTTTTATAGGTTCTAT
GGATAATTAGACAATTTTTGAAAGTATAGTATGCATATTAGTAATAGTAAGCAAGATG
ATTTTAAGCAACAAGTGTATGGACATATTGTAATAACTGAAAAATATATAAAGCTAGC
ACATTTAACATGTGATGTATAAGTTAAAGTTAAAGTCTGCTGCATATAAAGAAAGCCT
ATCTAATAGGGCTTTCCTAATTTCTGGCACCAATTCCTGAGCCTTTATGTTTGGACAA
CTCTCCTGCCTTATCAGAAAAACTGACCTCCAGGTCCAGAGAAAAGTGATTGGCTTTG

2.7.4.2. In-Silico PCR of Slected Primer for Exon 2:

>chr3:97767907+97768384 478bp GCTCCTTTCTGGTAATCAGCTT TGCAAATTATGGCTGAGGAT
GCTCCTTTCTGGTAATCAGCTTacactgtgtttactaagtgcaaagctac
attgacatagttttcacactattattatgtgtatttaaattattaccttt
ttttaatacacctaccaatattttccataacttaaggtgcctttgggtaa
tattttatttttcttaattgcagctggtttgtaaatatttgaatcacat
tatgggattgctagacagactttcagtcttgcttggcctgaagaagaagg
aggttcatgttttgtgccttgggctagataatagtggcaaaacgacgatc
attaacaaacttaaaccttcaaatgtaagtatctttgttagatgctttat
gtattttctgctactaaagaaaattaatgtgcagaattatgttatatgac
gttaaaaccgcatataatcacattaagatattctgttaacctttcagtac
ctttaagtATCCTCAGCCATAATTTGCA

2.7.4.3. FASTA Sequence of Selected Exon 5 of *ARL6* Gene:

TGAAAAAATTACTCATTTTTTAAAATTATGAAGTAGCAAGTCATTA
AATTTACTTTATTATCCAAATGAAAAGGTATTTTACATGAAATATG
ACTAATGATTAATATATTTATCAGTCATATAAACTAATAAGAAACA
ACACCAAAAATTCTAATGATATATGGACATAGGACATAAGGAGATA
ATACTTAGGTTAGGAAATGCCCATGGATAATAAACATGGAAAAATT
TATAAGTAAAGCTTTAATTTTTCTTTTTCTTTACATTACACAGAGA
AGGCCAAGCTATTATTTTTGTCATTGATAGTAGTGATAGATTAAGA
ATGGTTGTGGCCAAAGAAGAACTCGATACTCTTCTGAATCATCCAG
GTATGTGTGTGTCTTTCAGTCTGTATCTGTTCTTTGGGGTTCATTT
ATGTGTAATGTTTTGTTCTTTGGGTATTGCTTATACTATGTAATTA
TCTTTCATTTAAAATTTTTGAATTTAAAACATATATGTTATACAAA
ATTTATATTTTATGTACATCACCATATTTTGTAAATTTTGCATGCA
ATGTAAATGTAAAGCATTTTGCATTGTTTTTGGGGTGTCTAACATT
TATTTCCTCAAAAATAAAAATTCCCTTTTTTCTGATTACATAAATT
GTACCAAAGCAAAAAAAAAAAAAAAAAATCAGATGGAAAAATAAAATA
AAGATCTGTGTATTTCAACACCCATTAAAATTATTTGTTACCATAT
TTTCATGCATTTCTCTATGTGTATCTGAGTTTGTGTGTGTTTAATT
ACAGCATTTCTTACATATTTATTCATATATATATATATATATATCT
CCCAAAAATATCTGTAGGGTTGAGGACTGTGGGGACTATCATTGTT
ATTTACCAAAATGGAATCATATTCTTTACACTCTTCTGCATGTTTC
TATTCTCAGTTAACACTTAATGATGGATATCCTTCCAAATCAAATT
ATATATAATTATGTAGTTATTCTTTTTAATGATGCCTAATATGGTT

2.7.4.4. In-Silico PCR of Slected Primer for Exon 5:

>chr3:97784621+97785273 653bp TTGCATCGGTGTAATAGGGTTA AGACACCCCAAAAACAATGC
TTGCATCGGTGTAATAGGGTTAtggtattttaactcattttctcatattc
tgatattttaatgaaaaaattactcatttttaaaattatgaagtagcaa
gtcattaaatttactttattatccaaatgaaaaggtattttacatgaaat
atgactaatgattaatatatttatcagtcatataaactaataagaaacaa
caccaaaaattctaatgatatatggacataggacataaggagataatact
taggttaggaaatgcccatggataataaacatggaaaaatttataagtaa
agctttaatttttcttttttctttacattacacagagaaggccaagctatt
atttttgtcattgatagtagtgatagattaagaatggttgtggccaaaga
agaactcgatactcttctgaatcatccaggtatgtgtgtgtctttcagtc
tgtatctgttctttggggttcatttatgtgtaatgttttgttctttgggt
attgcttatactatgtaattatctttcatttaaaatttttgaatttaaaa
catatatgttatacaaaatttatattttatgtacatcaccatattttgta
aattttgcatgcaatgtaaatgtaaagcattttGCATTGTTTTTGGGGTG
TCT

2.7.5 Primer Dilutions:

The initial concentrations of the ordered primers were 100 picomole/ul, and subsequently these primers were diluted to achieve values of 10 picomole/ul.

2.7.6. Polymerase Chain Reaction:

The amplification of selected exons (Exon 2 and 5) of BBS3/ARL6 gene was done for six families through polymerase chain reaction that was done in 200ul capacity PCR tubes (Axygen,USA). The kit (Thermo-Scientific PCR Kit) was used for PCR amplification. Chemicals and volume used in PCR reaction is given in table.

Table 2.4: Chemicals used in PCR Mixture

Sr. No	Chemicals	Concentration	Volume (µl) for single PCR Reaction
1	DNTPs	2.5mM	2.5µl
2	MgCl2	25Mm	2µl
3	DNA	>1OOng/ul	2µl
4	Forward Primer	10 picomole/ul	0.5µl
5	Reverse Primer	10 picomole/ul	0.5µl
6	Taq. Buffer [(NH4)SO4]	10X	2.5µl
7	PCR Water		14.5 µl
8	Taq Polymerase	5U/ µl	0.5µl
	Total Volume		**25 µl**

Before placing the PCR tubes in the thermos cycler (Bio-Rad T100) for the PCR reaction, they were given a brief spin in the microfuge at 3000 rpm for one minute to mix the contents. The table below lists the temperature settings for the PCR reaction.

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	65-55 °C	45 sec	
Extension	72 °C	90 sec	
Final Extension	72 °C	10 min	1X
Hold	25 °C	∞	

2.7.6.1. Confirmation of PCR product By Running 2% Agarose GEL:

To verify the PCR results, a 2% agarose gel was created by combining 1g of agarose powder with 50ml of IX TBE buffer and then 2µl of Ethidium Bromide was added during gel preparation. Then these DNA samples were allowed to run for 20 min at 120V against a 2% agarose gel. To confirm the amplification of the targeted section of the BBS3/ARL6 Gene, the gel was then viewed using the Gel Documentation System (Cleaver Scientific Limited).

2.8. Purification of PCR Product

PCR product was purified by using purification kit (Thermo-Scientific). The procedure applied for purification in given below.

- PCR product was transferred to 2.5ml Eppendorf tubes.

- Our PCR product was almost 20µl so we took 100µl of Binding buffer that was 5 times the volume of PCR product. These were mixed properly.
- The mixture was moved to a labelled spin column that was attached to a collection tubes that were also labeled.
- The mixture was centrifuged for two minutes in a Beckman Coulter Microfuge at 13000 rpm.
- Each sample received 350µl of washing buffer, which was then centrifuged again for one minute at 13000 rpm. The filtrate was discarded.
- The PCR products were completely purified by repeating steps 5.
- The filtrate was completely discarded.
- Each sample received 13µl of elution buffer. Before usage, the elusion buffer was maintained in an incubator at 70°C.
- In Eppendorf tubes with pre-attached labels, the spin column tubes were put.
- The samples were maintained for two minutes at room temperature.
- For one minute, tubes were centrifuged at 13000 rpm.
- To verify the purity of the PCR products, samples were tested on 2% Agarose Gel.

2.9. Sequencing:

Sanger sequencing technique was applied for the sequencing of amplified PCR product. Approximately 10µl of purified PCR product present in each eppendorf tube along with 30µl forward and reverse desired primer(10picomole/µl) of selected exons for sequencing in separate tubes were labeled and sealed carefully and sent for commercial sequencing. To accomplish the sequencing reaction Big Dye Terminator chemistry (an automated ABI PRISM 3730 Genetic analyzer) was applied. The labeled DNA fragments were separated using the capillary electrophoresis technique and were detected using a

spectrum analyzer. For documentation purposes, each nucleotide (thymine, guanine, cytosine, and adenine) was labeled using a specific dye.

2.10. 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.jevi.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 35 families belonging from different parts of Pakistan were collected at Al-Shifa Hospital Rawalpindi. All families were having positive history of Retinitis Pigmentosa and at least two individual were diagnosed with RP. A detailed interview was conducted from all families after that, Bardet-Biedl families were extracted from these 35 families that were 6 in number. From each family proband was selected for mutation analysis in exon 2 and exon 5 of *ARL6* gene. All families possessing certain primary and secondary features of BBS that are listed in table 3.1 and 3.2. Out of these 6 families 5 were from Punjab province of Pakistan and one family was from Kotli AJK.

Table 3.1: Primary Symptoms Observed in Proband of each selected BBS Family

	BBS Features in Families Chosen For Genetic Analysis						
Sr. No	**Primary Features**	**Family ID/proband**					
		RP-04/V-1	**RP-06/II-5**	**RP-21/IV-1**	**RP-23/III-1**	**RP-43/IV-4**	**RP-46/V-9**
1	Rod-Cone Dystrophy	YES	YES	YES	YES	YES	YES
2	Polydactyly	YES	YES	NO	NO	YES	YES
3	Obesity	YES	YES	YES	YES	NO	YES
4	Learning Probelum	YES	YES	NO	YES	YES	YES
5	Hypogonadism	NO	NO	NO	NO	NO	NO
6	Renal Defects	NO	NO	NO	NO	NO	NO

Table 3.2: Secondary Symptoms Observed in BBS Families

	BBS Features in Families Chosen For Genetic Analysis						
	Secondary Features	**Family ID/proband**					
		RP-04/V-1	**RP-06/II-5**	**RP-21/IV-1**	**RP-23/III-1**	**RP-43/IV-4**	**RP-46/V-9**
1	Liver Disease	NO	NO	NO	NO	NO	NO
2	Heart Problem	NO	NO	NO	NO	NO	NO
3	Hearing Issue	NO	YES	YES	YES	NO	NO
4	Dental Issues	NO	NO	NO	YES	NO	NO
5	Speech Problems	NO	NO	NO	NO Brother/YES	NO	NO
6	Developmental Delay	NO	NO	NO	NO	NO	NO
7	Diabetes Mellitus	NO	NO	NO	NO	NO	NO
8	Family Members with BBS	YES	YES	YES	YES	YES	YES

3.1.1. Family RP O4:

The RP family 04 belongs from Jhang Pakistan. This family consist V generations having 25 members out of which 20 are alive and five are deceased. Four male members (IV-5, V-1, V-4, and V-5) are diagnosed with RP one of them is deceased (IV-5) as shown in fig 3.1. The primary and secondary features of BBS possessed by the proband V-1 are listed in table 3.1 and 3.2. This family shows an autosomal recessive mode of inheritance but further genetic analysis is required to confirm the pattern of inheritance. Affected members (V-4 and V-5) also possess BBS signs.

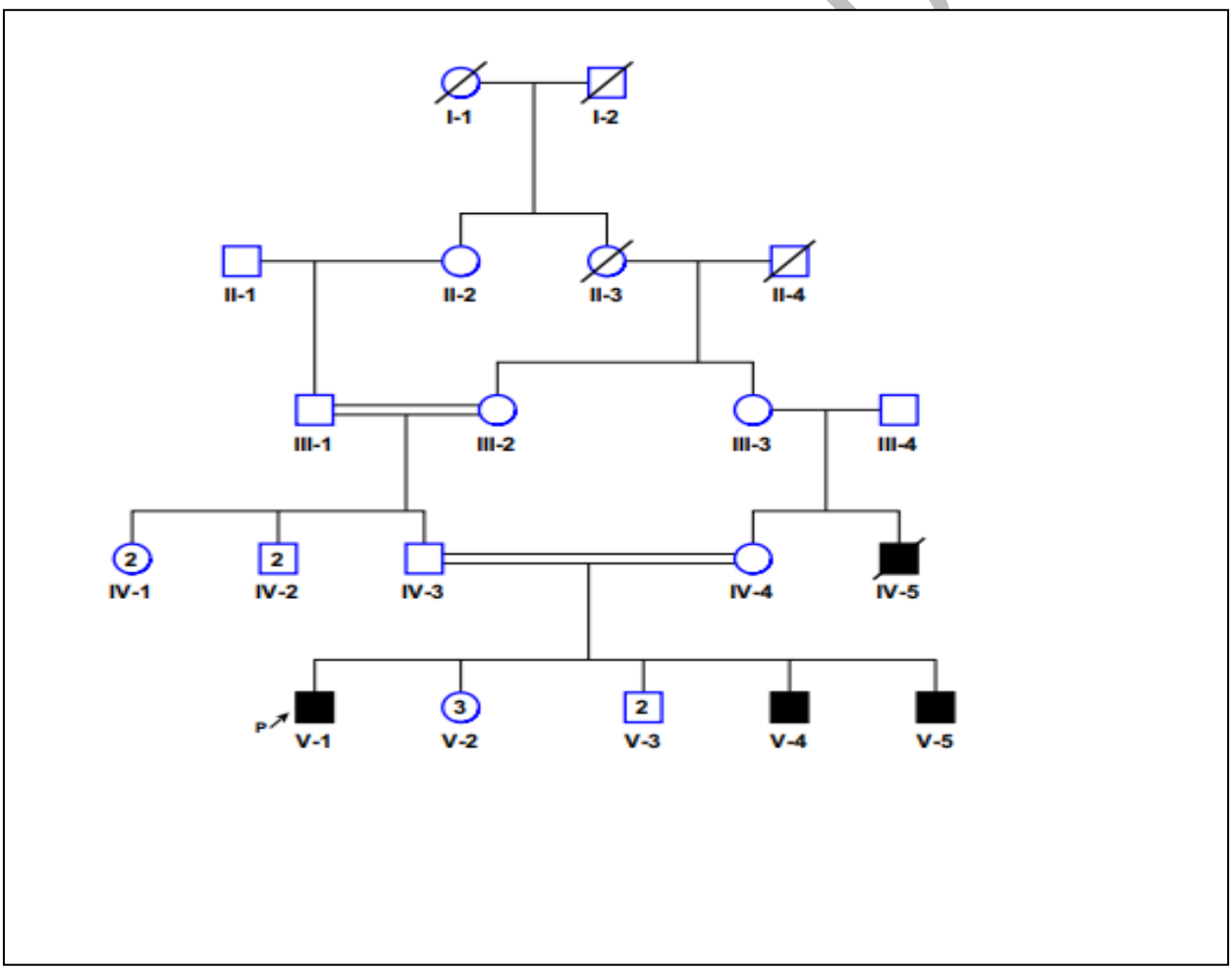

Fig. 3.1: Pedigree of RP-04 Family.

3.1.2. Family RP O6:

The RP family 06 belongs from Jhang Pakistan. This family consist IV generations having 21 members all are alive. Two female members (III-5 and IV-4) have complete symptoms of BBS that are shown in pedigree fig 3.2. The primary and secondary features of BBS possessed by the proband III-5 are listed in table 3.1 and 3.2. At the time of recruitment the age of proband was 8 year. This family shows an autosomal recessive mode of inheritance but further genetic analysis is required to confirm the pattern of inheritance.

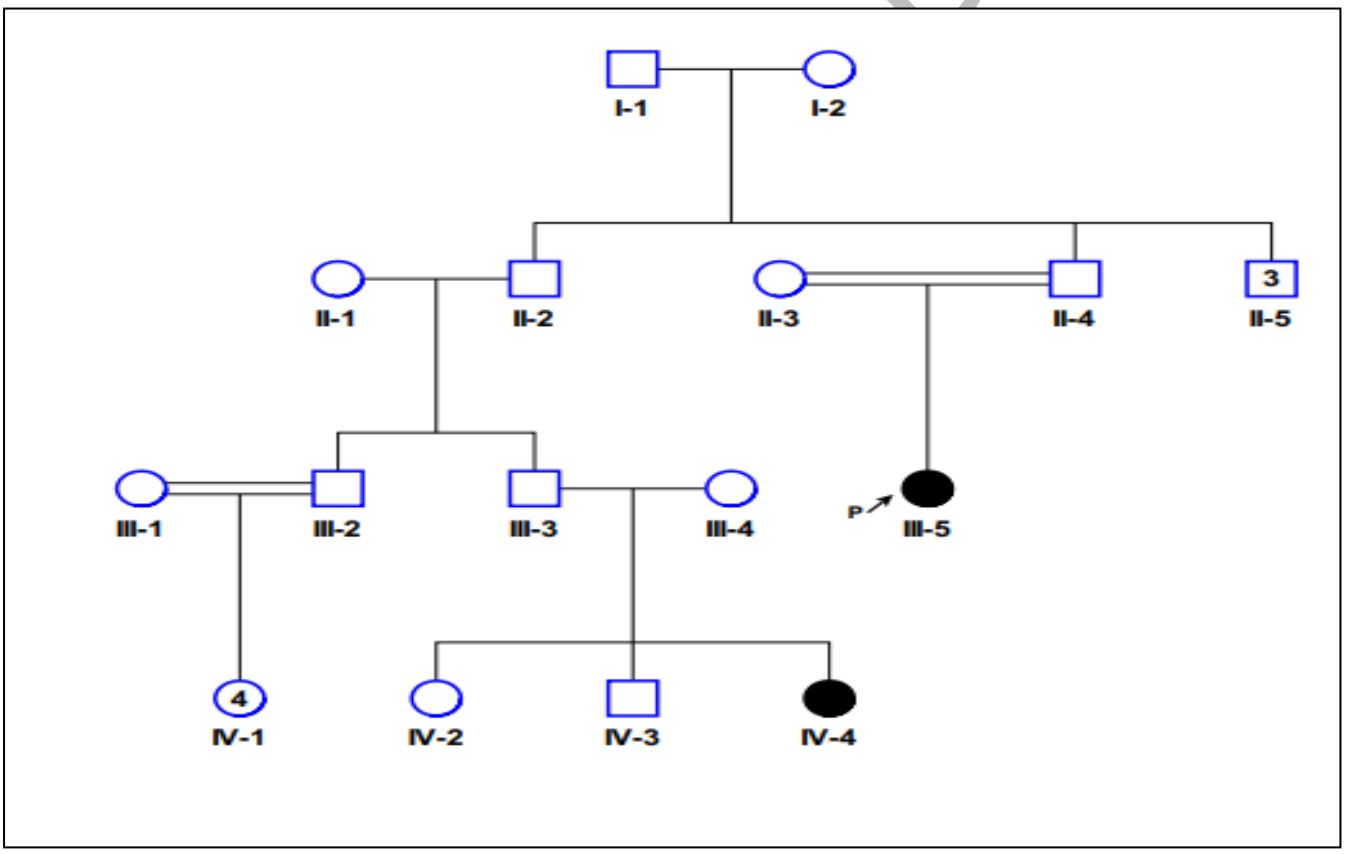

Fig. 3.2: Pedigree of RP-06 Family.

3.1.3. Family RP-21:

The RP family 21 belongs from Kotli AJK. This family consist IV generations having 36 members out of which 23 individuals are alive and 13 are deceased. Two male members (IV-1 and IV-2) have complete symptoms of BBS that are shown in pedigree fig 3.3. Three members of the family have polydactyly (III-3, III-1, IV-3) and one member (IV-4) has tongue problem and other symptoms related to BBS but until now they have not consulted a doctor regarding diagnosis. This family shows an autosomal recessive pattern of inheritance. IV-1 proband of the family 21was selected for genetic analysis. At the time of enrolment the age of proband IV-1 was about 26 years and the other affected member IV-2 age was about 24 years.

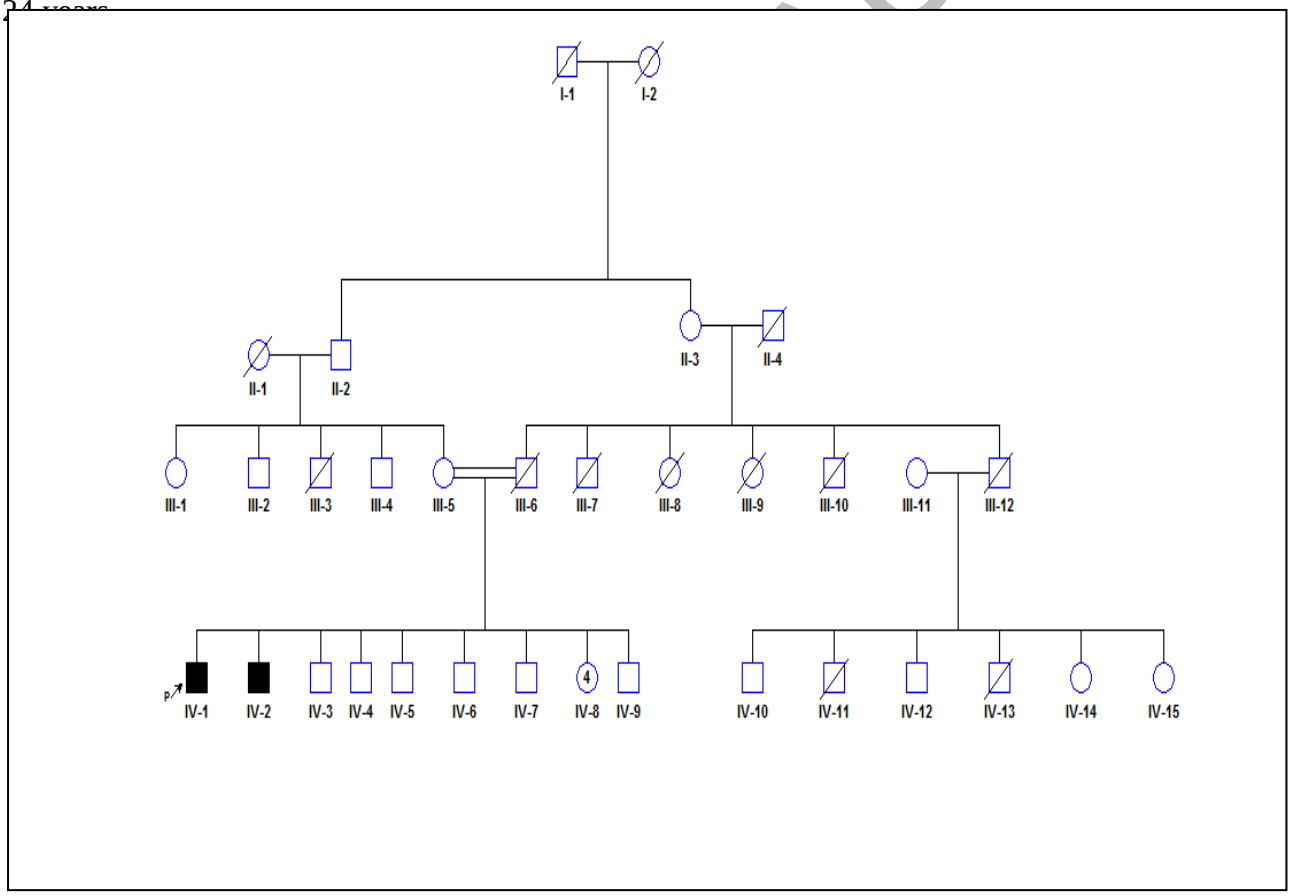

Fig. 3.3: Pedigree of RP-21 Family.

3.1.4. Family RP-23:

The family RP 23 consists of V generations having 46 members out of which 41 are alive and 5 are deceased. This family consists of six affected members and this belongs from Rawalpindi. This family consists of six affected members, five are males (III-1, III-4, IV-2, IV-5 and V-1) and one is female (IV-6). All the affected members are diagnosed with RP and these members also possess the signs of BBS. After conducting a detailed interview with proband III-1, the proband was assigned BBS and its genetic analysis was performed because it was possessing BBS primary and secondary features as mentioned in table 3.1 and 3.2. At the time of enrolment the age of proband was 36 years. This family depicts an X-linked dominant pattern of inheritance but it is not confirmed and further genetic analysis is required to confirm the pattern of inheritance.

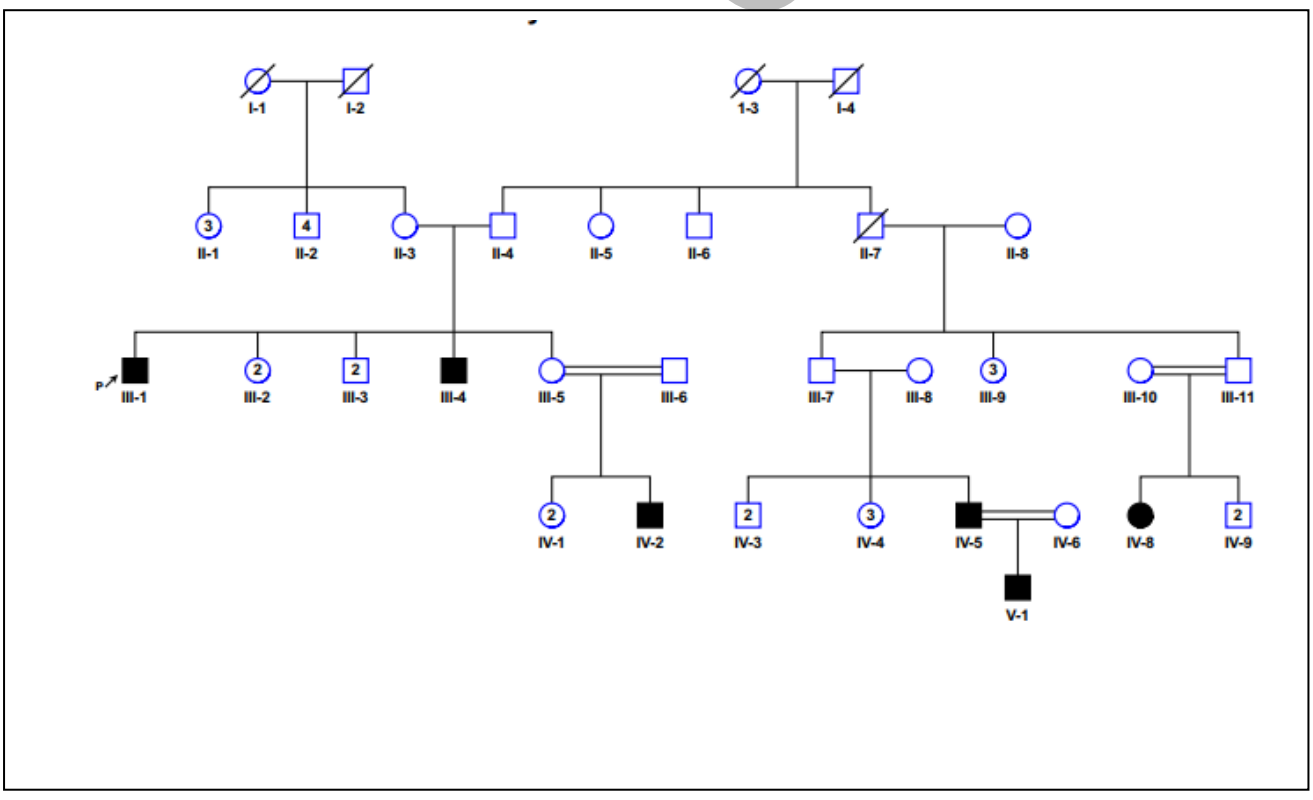

Fig. 3.4: Pedigree of RP-23 Family.

3.1.5. Family RP-43:

The family RP 43 consists of IV generations having 25 members out of which 23 are alive and 2 are deceased. This family consists of three affected members and this belongs from Gujrat Pakistan. This family consists of three affected members; all the affected members are females (IV-2, IV-4, and IV-5). All the affected members are diagnosed with RP and these members also possess the signs of BBS. After conducting a detailed interview with proband IV-4, the proband was assigned BBS and its genetic analysis was performed because it was possessing BBS primary and secondary features as mentioned in table 3.1 and 3.2. At the time of enrolment the age of proband IV-4 was 20 years and the age of other affected members was 25 (IV-2) and 24(IV-5) years respectively. This family shows an autosomal recessive mode of inheritance but further genetic analysis is required to confirm the pattern of inheritance.

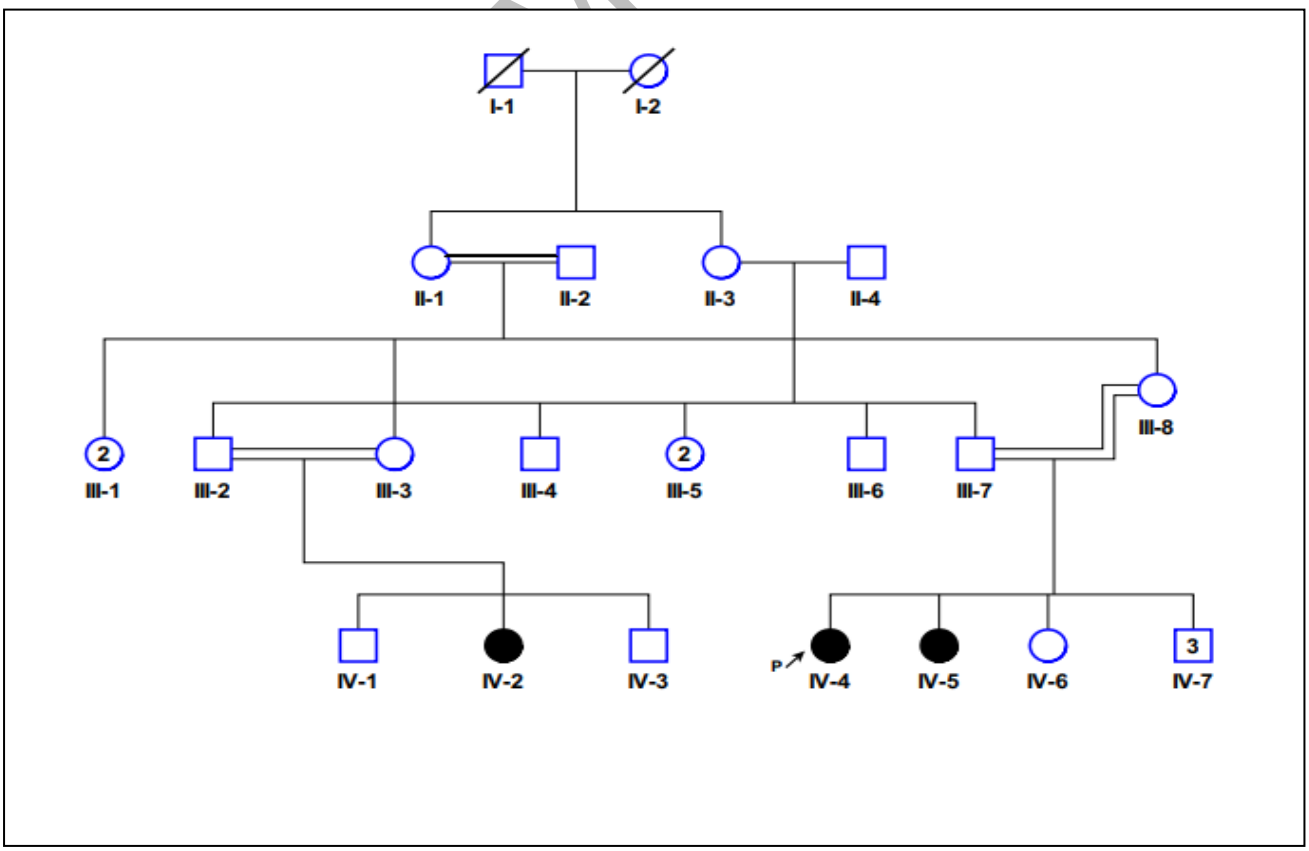

Fig. 3.5: Pedigree of RP-43 Family.

3.1.6. Family RP-46:

The family RP 46 consists of V generations having 36 members out of which 21 are alive and 15 are deceased. This family consists of six affected members and this belongs from Gujar Khan Pakistan. This family consists of six affected members; all the affected members are males (V-1, V-3, V-5, V-6 and V-9). All the affected members are diagnosed with RP and these members also possess the signs of BBS. After conducting a detailed interview with proband V-9, the proband was assigned BBS and its genetic analysis was performed because it was possessing BBS primary and secondary features as mentioned in table. At the time of enrolment the age of proband V-9 was 22 years and the age of other affected members was 25 (V-7), 35(V-6), 28(V-5), 40(V-3), and 35(V-1) years respectively. This family shows an X-linked recessive mode of inheritance but further genetic analysis is required to confirm the

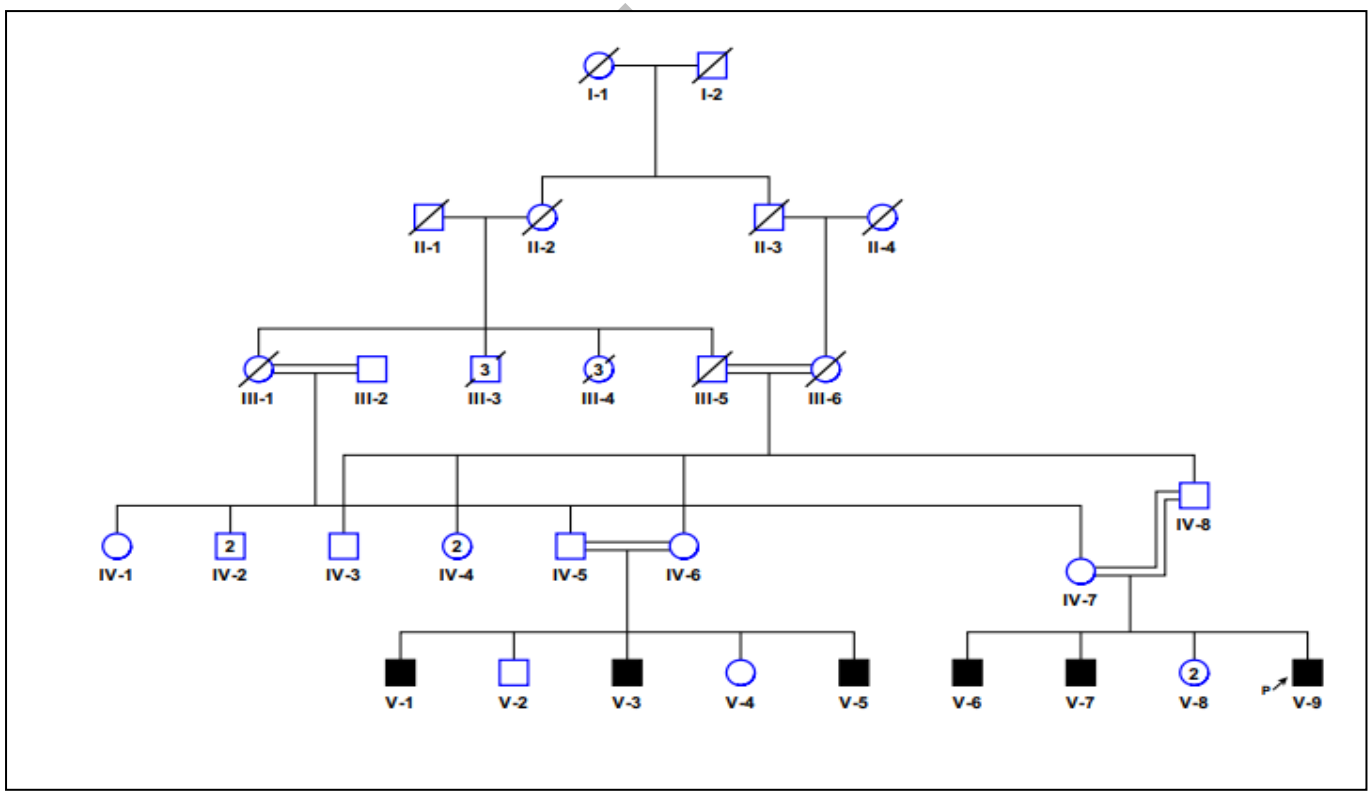

Fig. 3.6: Pedigree of RP-46 Family.

3.2. DNA Isolation:

The DNA was isolated from each blood sample using phenol chloroform method. The mean concentration of isolated DNA was up to 146.33 ng/µl with purity value of 1.69 for each isolated sample.

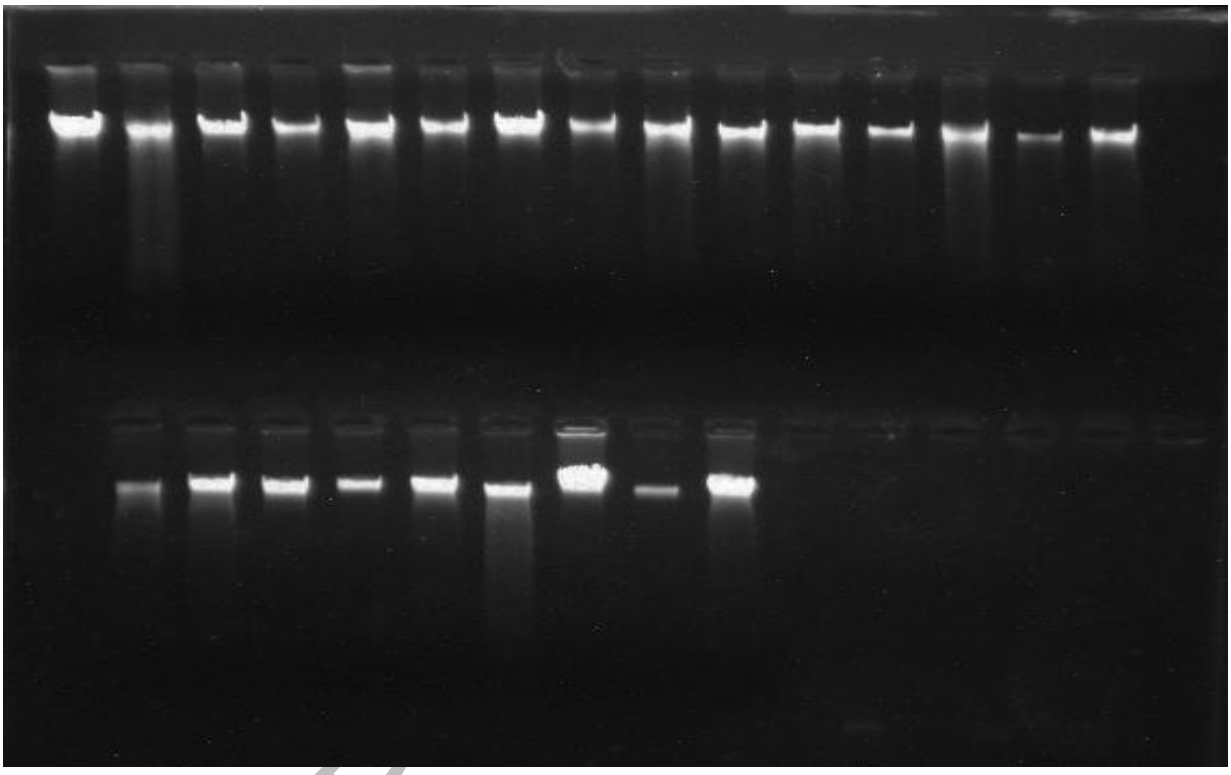

Fig. 3.7: DNA Isolated from BBS Families.

3.3. Polymerase Chain Reaction:

Polymerase Chain Reaction (PCR) was performed to amplify the selected exon 2 and exon 5 of *ARL6* gene of proband of BBS families. The primer used for the amplification of exon 2 has product size of 478 bp while the primer used for the amplification of exon 5 has product size of 653 bp. The confirmation of amplification was done by running 2% agarose gel electrophoresis and visualizing the gel in gel documentation system (Cleaver Scientific Limited) as shown in fig 3.8.

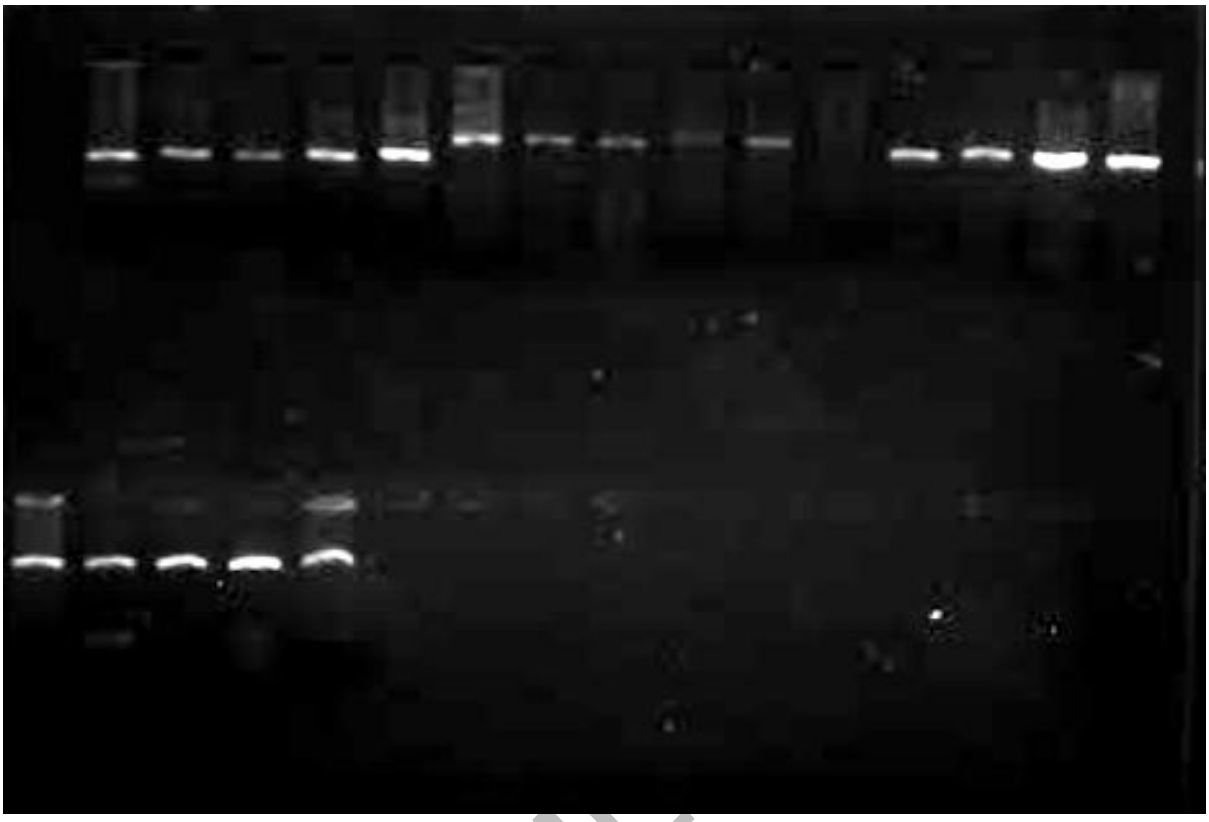

Fig.3.8: PCR Amplification results on 2% Agarose gel

3.4. PCR Product Purification:

For the purification of PCR product, a purification kit (Thermo-Scientific) was used. All the PCR product of probands was purified and 13µl purified PCR products were obtained. The confirmation of amplification was done by running 2% agarose gel electrophoresis and visualizing the gel in gel documentation system (Cleaver Scientific Limited) as shown in fig 3.9.

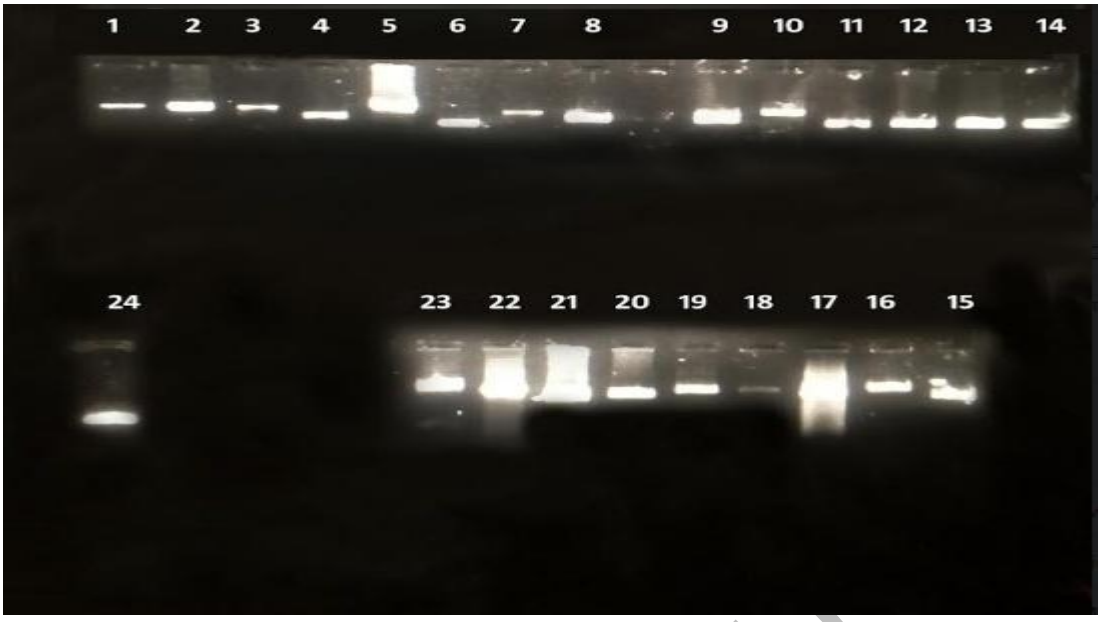

Fig.3.9: Confirmation of Purified PCR product.

3.5. Sanger 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. To accomplish the sequencing reaction Big Dye Terminator chemistry (an automated ABI PRISM 3730 Genetic analyzer) was applied. The labeled DNA fragments were separated using the capillary electrophoresis technique and were detected using a spectrum analyzer. For documentation purposes, each nucleotide (thymine, guanine, cytosine, and adenine) was labeled using a specific dye.

3.6.Genetic Analysis

Mutations in *ARL6* gene are known to cause Bardet-Biedl syndrome in different popullations globally.Exon 2 and 5 of *ARL6* gene (Ensemble Transcript I.D: ENST00000463745.6) were selected for genetic analysis of choosen families. *ARL6 having* Ensemble Transcript I.D: ENST00000335979.6 was used as standard for sequence alignment. All the analyzed sequences are listed below.

Family RP-04

Exon 2 Analyzed Sequence

Sanger sequencing of exon 2 of *ARL6* gene of selected proband of family shows no mutation or change in the DNA sequence.

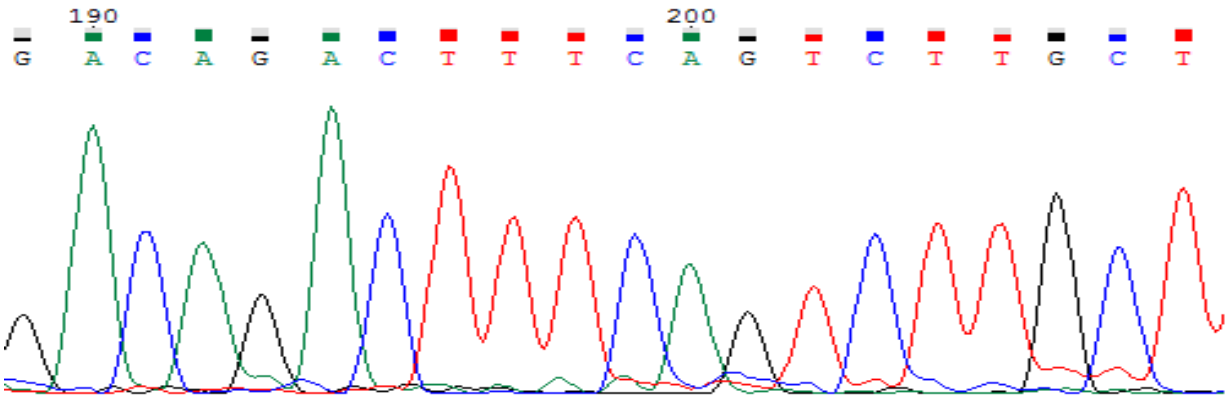

Fig.3.10 : Sequencing Chromatogram: Showing no change in exon 2 of proband of RP-04.

Family RP-04

Exon 5 Analyzed Sequence

Sanger sequencing of exon 5 of *ARL6* gene of selected proband of family shows no mutation or change in the DNA sequence.

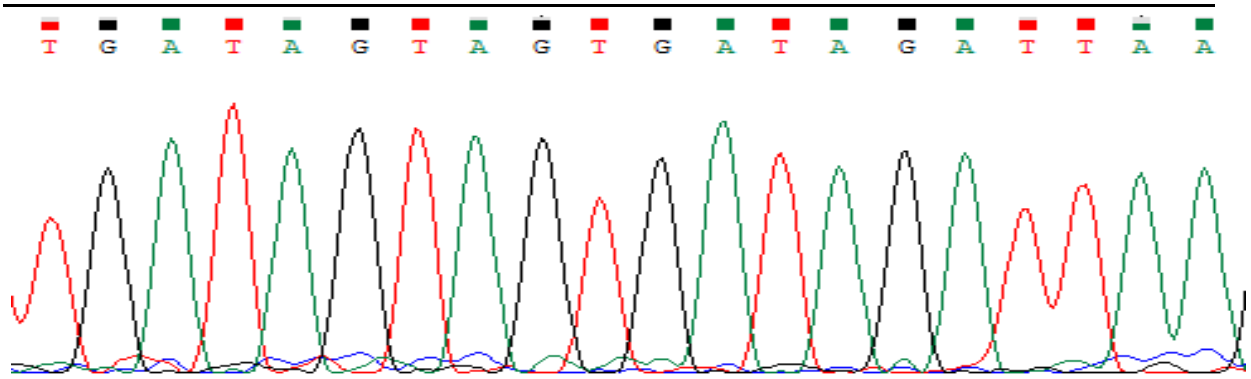

Fig.3.11: Sequencing Chromatogram: Showing no change in exon 5 of proband of RP-04.

Family RP-06

Exon 2 Analyzed Sequence

Sanger sequencing of exon 2 of *ARL6* gene of selected proband of family shows no mutation or change in the DNA sequence.

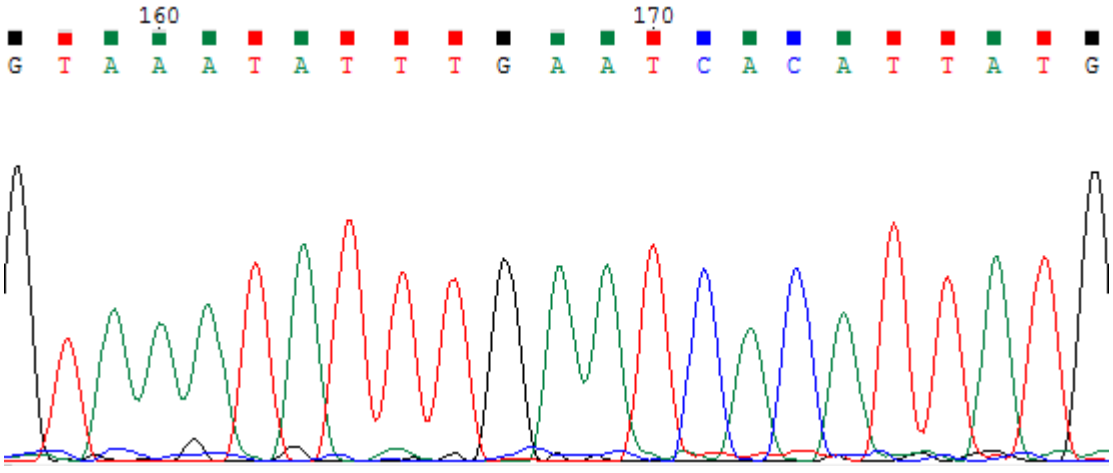

Fig.3.12: Sequencing Chromatogram: Showing no change in exon 2 of proband of RP-06.

Family RP-06

Exon 5 Analyzed Sequence

One polymorphism is found in intronic region at position g.20299-20300insC during analysis of exon 5 sequence of ARL6 gene of RP family 06.

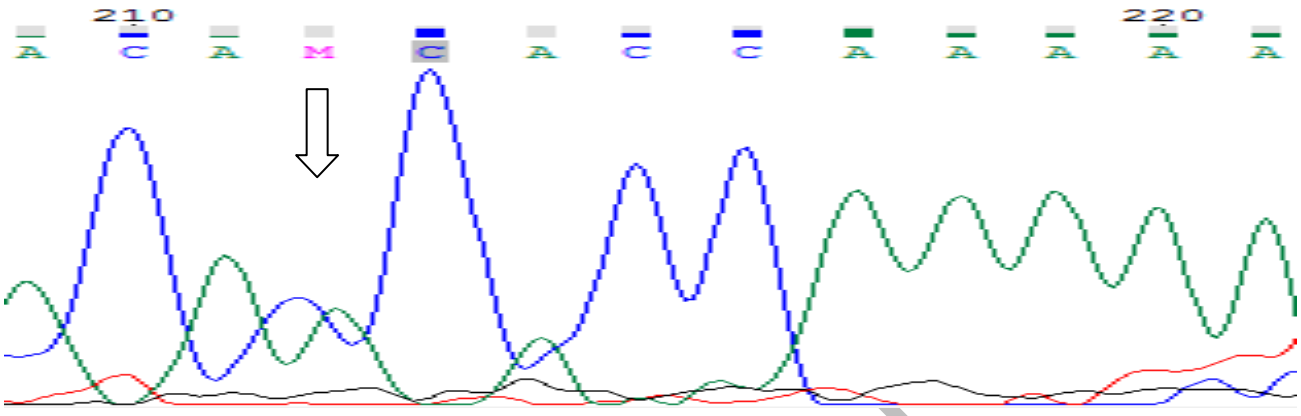

Fig.3.13: Sequencing Chromatogram: Showing intronic polymorphism at position g.20299-20300insC in chr3:97503663-97503664insC in exon 5 of *ARL6* gene of RP 06 family.

Family RP-21

Exon 2 Analyzed Sequence

Sanger sequencing of exon 2 shows no mutation or polymorphism in selected proband of family RP21P1.

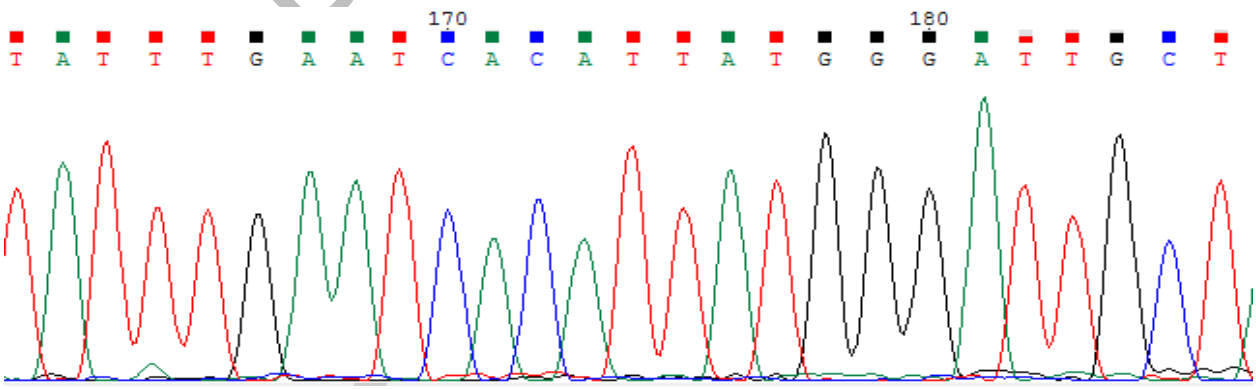

Fig.3.14: Sequencing Chromatogram: Showing no change in exon 2 of proband of RP-21.

Family RP-21

Exon 5 Analyzed Sequence

Sanger sequencing of exon 5 shows mutation c.266C>A at chr3:97503810C>A, mutation c.266-267insA at chr3:97503810-97503811insA and mutation c.304-305insGG at chr3:97503848-97503849insGG in exon 5 of ARL6 gene of RP21 family

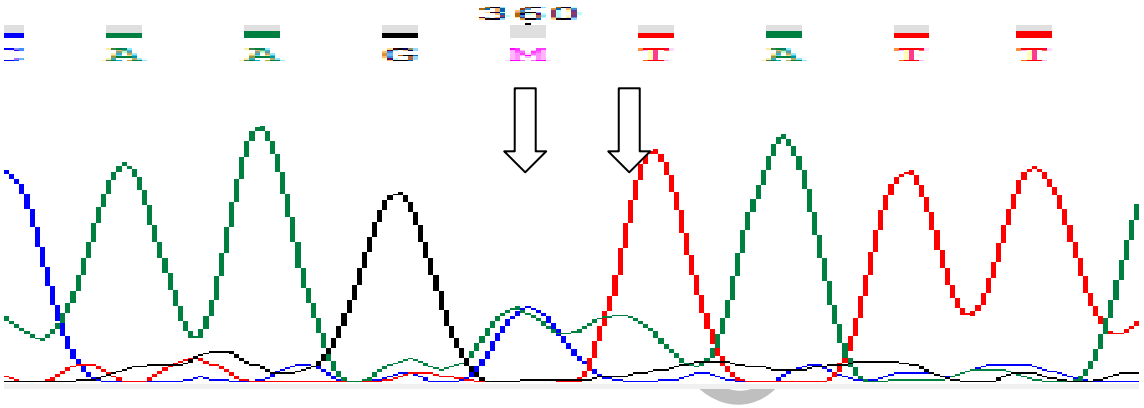

Fig.3.15: Sequencing Chromatogram: Showing mutation c.266C>A at chr3:97503810C>A and mutation c.266-267insA at chr3:97503810-97503811insA in exon 5 of ARL6 gene of RP 21 family.

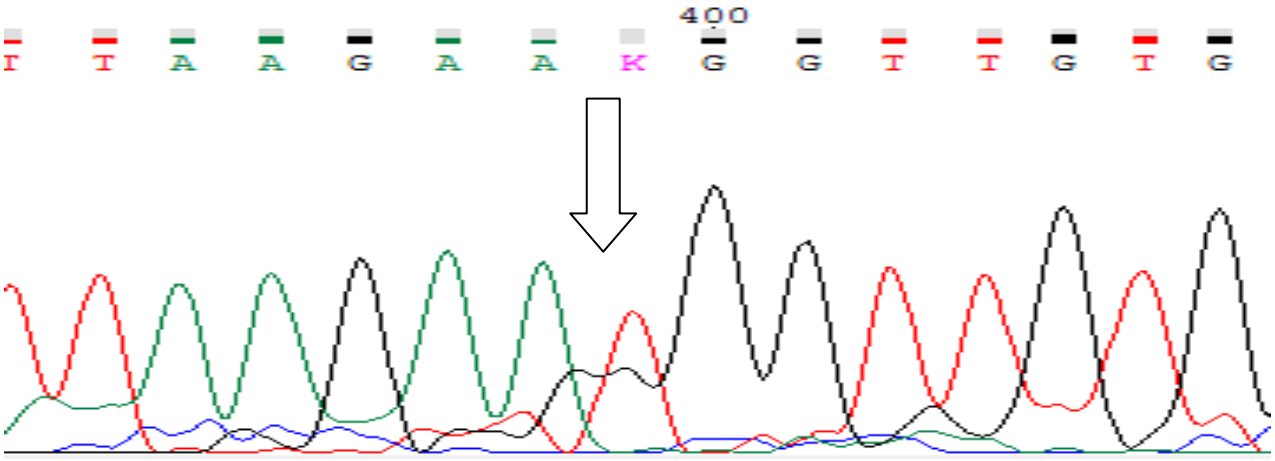

Fig.3.16: Sequencing Chromatogram: Showing mutation c.304-305insGG at chr3:97503848-97503849insGG in exon 5 of ARL6 gene of RP21 family.

Family RP-23

Exon 2 Analyzed Sequence

Sanger sequencing of exon 2 of *ARL6* gene of selected proband of family shows no mutation or change in the DNA sequence.

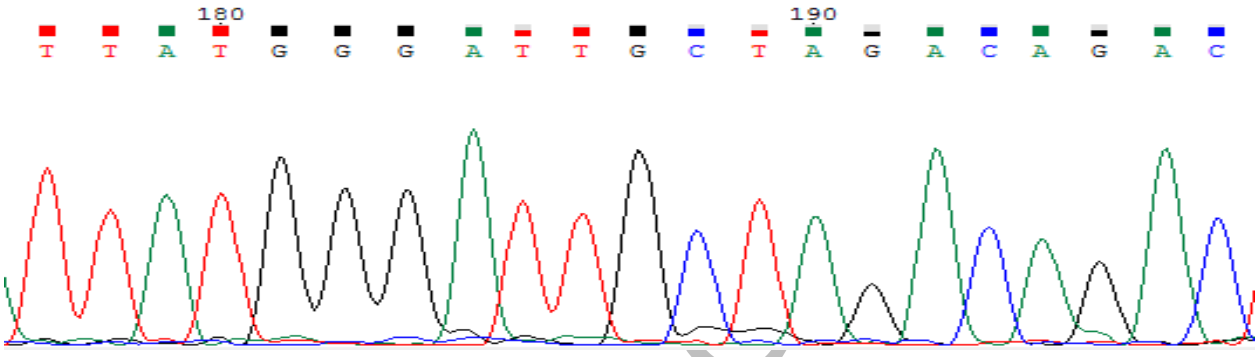

Fig.3.17: Sequencing Chromatogram: Showing no change in exon 2 of proband of RP-23.

Family RP-23

Exon 5 Analyzed Sequence

Sanger's sequencing depicts polymorphism at position (g.20350-20351insCG in chr3:97503714_97503715insCG),polymorphism at position (g.20388-20389insC in chr3:97503752-97503753insC) and intronic polymorphism g.20389-20390insTG in chr3:97503753-97503754insTG in exon 5 of *ARL6* gene of RP 23 family.

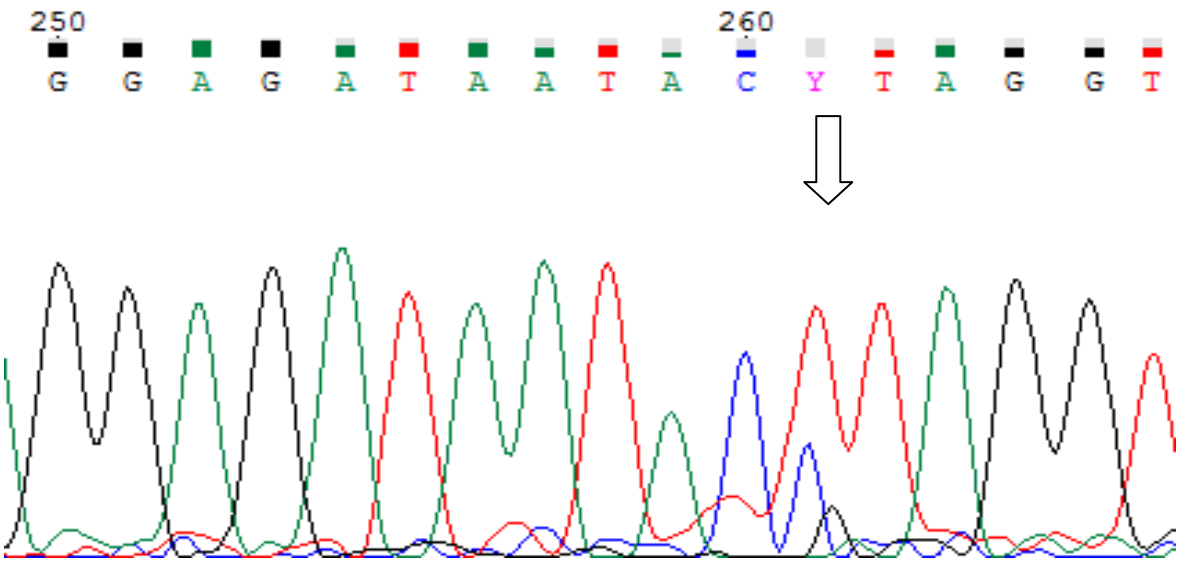

Fig.3.18: Sequencing Chromatogram: Showing polymorphism at position g.20350-20351insCG in chr3:97503714_97503715insCG in exon 5 of *ARL6* gene of RP 23 family.

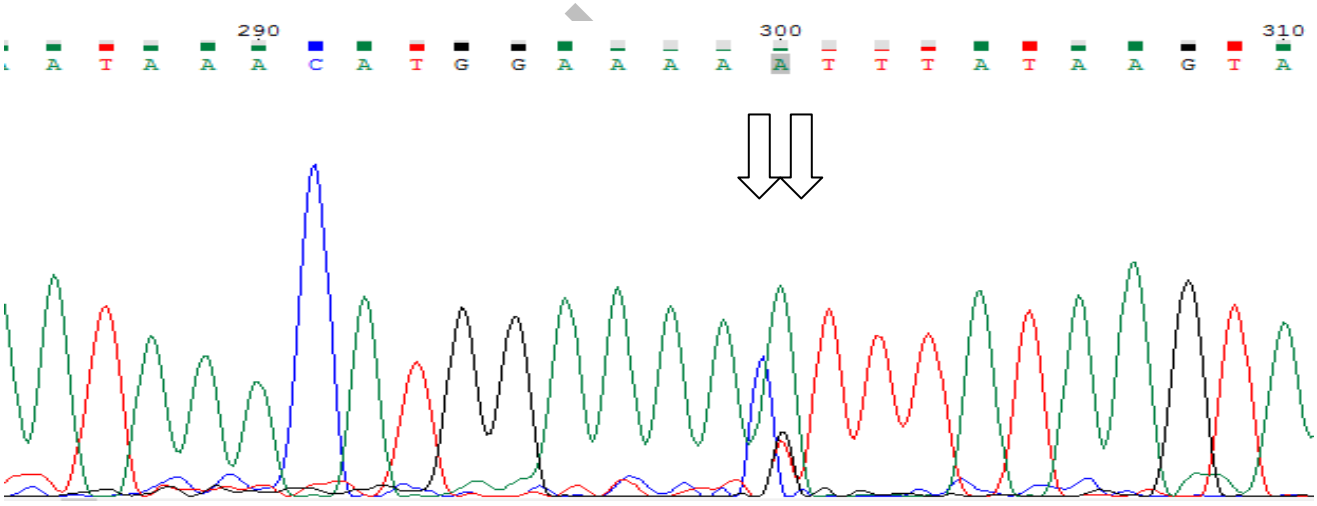

Fig.3.19: Sequencing Chromatogram: Showing intronic polymorphism at position g.20388-20389insC in chr3:97503752-97503753insC and another intronic polymorphism at position g.20389-20390insTG in chr3:97503753-97503754insTG in exon 5 of *ARL6* gene of RP 23 family.

Family RP-43

Exon 2 Analyzed Sequence

Sanger sequencing of exon 2 of *ARL6* gene of selected proband of family shows no mutation or change in the DNA sequence.

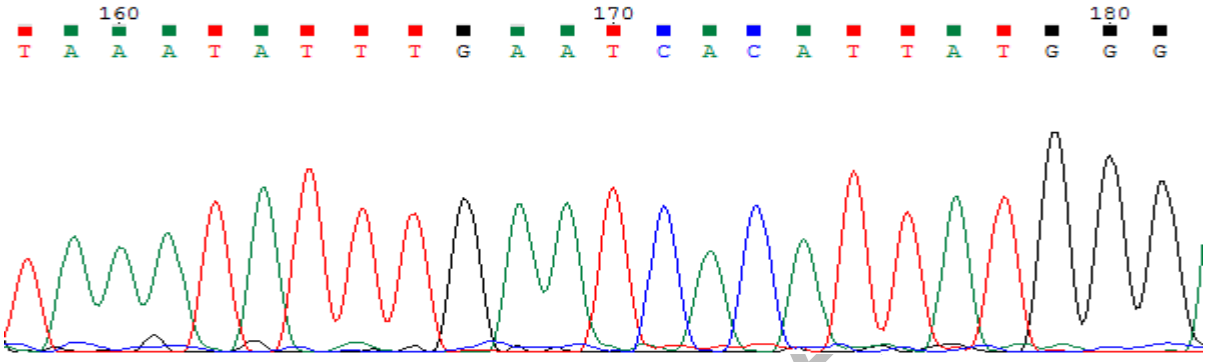

Fig.3.20: Sequencing Chromatogram: Showing no change in exon 2 of proband of RP-43.

Family RP-43

Exon 5 Analyzed Sequence

Sanger sequencing of exon 5 shows mutation c.281T>C, p.I94T at chr3:97503825T>C in exon 5 of *ARL6* gene of RP 43 family.

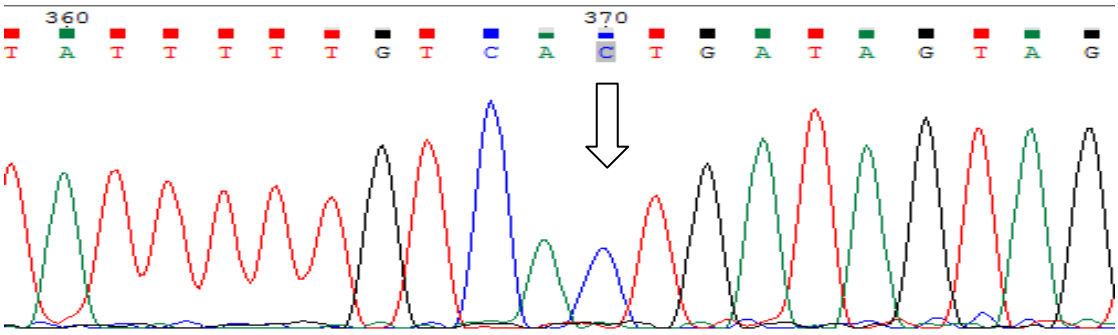

Fig.3.21: Sequencing Chromatogram: showing mutation c.281T>C (rs438186) at chr3:97503825T>C in exon 5 of ARL6 gene of RP 43 family.

Family RP-46

Exon 2 Analyzed Sequence

Sanger sequencing of exon 2 of *ARL6* gene of selected proband of family shows no mutation or change in the DNA sequence.

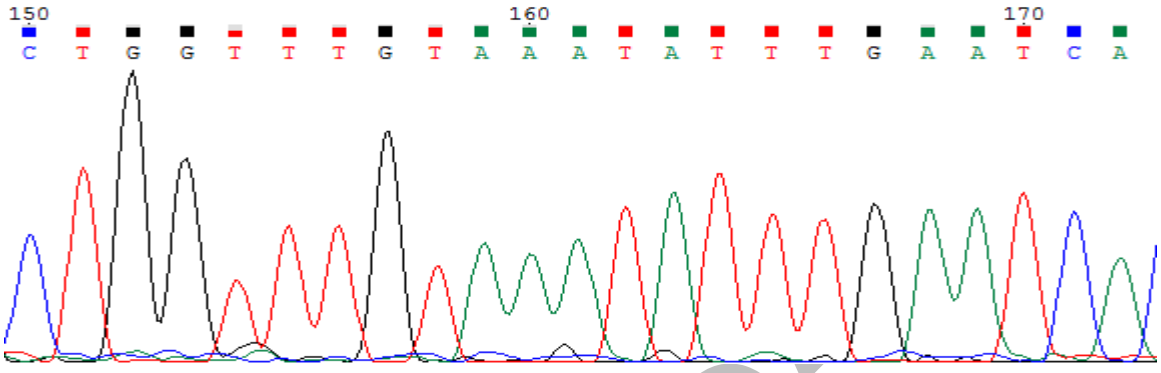

Fig.3.22: Sequencing Chromatogram: Showing no change in exon 2 of proband of RP-46.

Family RP-46

Exon 5 Analyzed Sequence

One polymorphism is found in intronic region at position g.20299-20300insC in chr3:97503663_97503664insC during analysis of exon 5 sequence of *ARL6* gene of RP family 46.

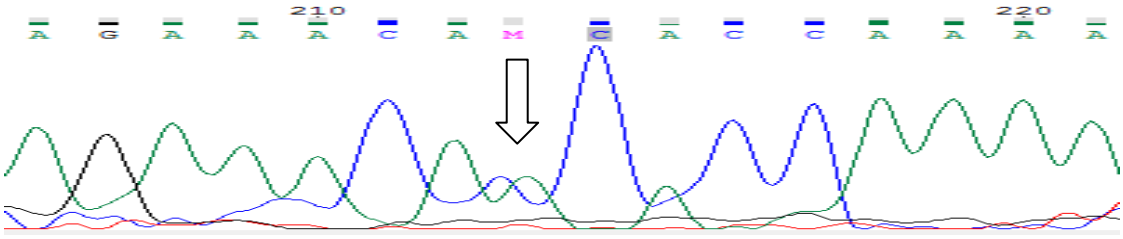

Fig.3.23: Sequencing Chromatogram: Showing polymorphism in intronic region at position g.20299-20300insC in chr3:97503663_97503664insC in exon 5 sequence of *ARL6* gene of RP family 46.

Table 3.3: Identified Polymorphism and Mutations in Enrolled BBS Families.

Exon No	Genomic Location	Physical Location	AA Change	rs. ID	Zygosity	Mutation Taster	Status
5	g.20299-20300insC	chr3:97503663-97503664insC	N/A	N/A	Homozygous	Polymorphism	**Novel**
5	c.266C>A	chr3:97503810C>A	p.A89D	N/A	Heterozygous	Mutation	**Novel**
5	c.266-267insA	chr3:97503810-97503811insA	I90Yfs*6	N/A	Homozygous	Mutation	**Novel**
5	c.304-305insGG	chr3:97503848-97503849insGG	M102Rfs*13	N/A	Homozygous	Mutation	**Novel**
5	g.20350-20351insCG	chr3:97503714_97503715insCG	N/A	N/A	Homozygous	Polymorphism	**Novel**
5	g.20388-20389insC	chr3:97503752-97503753insC	N/A	N/A	Homozygous	Polymorphism	**Novel**
5	g.20389-20390insTG	chr3:97503753-97503754insTG	N/A	N/A	Heterozygous	Polymorphism	**Novel**
5	c.281T>C	chr3:97503825T>C	p.I94T	rs438186	Homozygous	Mutation	**Reported**
5	g.20299-20300insC	chr3:97503663_97503664insC	N/A	N/A	Homozygous	Polymorphism	**Novel**

Chapter No. 4

Discussion

The aim of current study was to depict the demographic and clinical characteristics of BBS affected cases in our local population and to perform molecular analysis of *ARL6* gene to identify sequence variants. For this purpose a total of six families were studied in detail based on clinical presentation in accordance with reported BBS phenotype. BBS is a syndromic condition in which multiple varying disease symptoms are reported in articles observed among different patients so the diagnosis depends on primary and secondary symptoms (P. Beales *et al*., 1999; Forsythe & Beales, 2013; M'hamdi *et al*., 2014). The diagnosis of BBS is mainly dependent on seminial study of Beals *et al*. [1999] and mainly according to phenotype(Forsythe & Beales, 2013). The appearance of symptoms of BBS are related to age, in early age only few symptoms are apparent other symptoms evolve during or after first decade of life, most individuals having polydactyly at birth appears healthy but at later stages of life they are diagnosed with BBS(P. Beales *et al*., 1999). RCD mostly RP with macular involvement is the most frequent diagnostic sign triggering an examination for BBS and it is reported in 93% BBS patients (P. Beales *et al*., 1999). Patients should be given a BBS diagnosis if they exhibit at least four out of the disease's six major symptoms as mentioned in table 3.1. Two secondary features are necessary to confirm the presence of BBS if only three primary traits are found. This diagnosis criteria primarily characterize BBS as a clinical entity and it cannot adequately describes a person with disease attenuated form or gene-specific manifestations of BBS (Estrada-Cuzcano *et al*., 2012; Pawlik *et al*., 2010).Among six families enrolled in this study, five families (RP 04, 06, 23, 43, 46) were fulfilling the above mentioned BBS diagnosis criteria but family RP 21 was not fulfilling the BBS clinical diagnosis criteria. Previously Estrada-Cuzcano, Koenekoop *et al*, [2012],

Pawlik, Mir *et al*,[2010], and Cannon, Clayton-Smith *et al*,[2008] has reported mutations in BBS genes in those families that were not fulfilling the clinical diadnostic criteria of BBS(Cannon, Clayton-Smith, Beales, & Lloyd, 2008; Estrada-Cuzcano *et al*., 2012; Pawlik *et al*., 2010). In these families only one symptom such as obesity or only a few BBS symptoms were appearent that were not enough for clinical diagnostic criteria of BBS. This depicts that clinical diagnostic criteria of BBS is not stringent for molecular analysis of BBS syndrome. Therefore, the family RP 21, in which affected cases are having RP phenotype along with few other features i.e., polydactyly in individuals IV-3, III-1 and III-3 and tongue probelum in individual IV-4 was also selected for molecular analysis.

Autosomal recessive mode of inheritance was observed in BBS syndrome by initial gene discovery studies further more research complicated the genetics of BBS and prevailed incomplete penetrance and triallelic inheritance (Katsanis *et al*., 2001). Studies have shown individuals with biallelic gene mutation carriers for BBS that were healthy at investigation suggesting incomplete penetrance (Abu-Safieh *et al*., 2012). Studies also showed BBS patients with homozygous mutation in one gene and heterozygous mutation in other gene for BBS suggesting triallelic inheritance (P. L. Beales *et al*., 2003; Estrada-Cuzcano *et al*., 2012; Katsanis *et al*., 2001; Kwitek-Black *et al*., 1993; Leppert *et al*., 1994; Young *et al*., 1999).Experimental evidences reveals dominant negative effect by some BBS mutations affecting other gene functioning (Zaghloul *et al*., 2010). Families included in this study phenotypicalley exhibited autosomal recessive, X-linked dominant and X-linked recessive pattern of inheritance. Phenotypicalley almost 66.6% families were possessing autosomal recessive mode of inheritance, 16.6% were possessing X-linked dominant and 16.6% were possessing X-linked recessive pattern of inheritance. Further genetic study is required for the confirmation of mode of inheritance of families. Prevalence frequency of BBS in Pakistan is still not reported but all reported BBS mutat ions in Pakistan in are listed in table 1.3. *ARL6* and *BBS10*

are most mutated genes in Asia. In India by different studies *ARL6* and *BBS10* are proposed as mutational hotspot for BBS syndrome(Chandrasekar *et al*., 2018). Reports on mutations in BBS genes in Pakistan from Chen, Smaoui *et al*, [2011], Khan, Ullah *et al*, [2013], Maria, Lamers *et al*, [2016], Agha, Iqbal *et al*, [2013], Ullh, Umair *et al*, [2017] and Khan, Ullah *et al*, [2013] studies depicts that *ARL6* and *BBS10* are most mutated BBS genes in Pakistan and these are mutational hotspot in Pakistan and these are listed in table 1.3. In present study on the basis of availablity of funding only exon 2 and 5 of *ARL6* gene were selected for genetic analysis.

Sanger sequencing of exon 2 and 5 of *ARL6* gene of six familes were done and 9 varients were found, no varient was observed in exon 2 of ARL6 gene of selected families all nine varients were observed in exon 5 of ARL6 gene and among these 9 varients 5 were polymorphism and 4 were mutations. Out of 4 disease causing mutations 3 are novel and 1 mutation is previously reported. All the observed polymorphisms are not previously reported and out of five polymorphisms 4 are homozygous and one is hetrozygous. Out of 4 mutations 3 are homozygous while one is hetrozygous. Polymorphism is found in intronic region at position g.20299-20300insC during analysis of exon 5 sequence of ARL6 gene of RP family 06 and this was not reported by the previous studies. Sanger sequencing of exon 5 shows mutation c.266C>A at chr3:97503810C>A, mutation c.266-267insA at chr3:97503810-97503811insA and mutation c.304-305insGG at chr3:97503848-97503849insGG in exon 5 of ARL6 gene of RP21 family and all these mutations are novel. Sanger's sequencing depicts polymorphism at position (g.20350-20351insCG in chr3:97503714_97503715insCG), polymorphism at position (g.20388-20389insC in chr3:97503752-97503753insC) and polymorphism g.20389-20390insTG in chr3:97503753-97503754insTG in exon 5 of *ARL6* gene of RP 23 family all these polymorphisms are not previously reported.

Sanger sequencing of exon 5 shows mutation c.281T>C, p.I94T (rs438186) at chr3:97503825T>C in exon 5 of *ARL6* gene of RP 43 family and this is previously first time reported mutation from Pakistan by (Khan, S., *et al*, [2013]). According to mutation taster

(http://www.mutationtaster.org/) this mutation leads to a change of Isoluceine at position 94 with Threonine (p.I94T). Polymorphism is found in intronic region at position g.20299-20300insC in chr3:97503663_97503664insC during analysis of exon 5 sequences of *ARL6* gene of RP family 46 and this is not previously reported.

All the genetic variations observed in selected regions (exon 2 and 5) of *ARL6* gene of selected families RP (04, 06, 21, 23, 43, and 46) are not confirmed by segregation analysis. For the conformation of variations genotyping of parents and other family members would be performed and segregation analysis would be checked. Again Sanger sequencing of selected regions (exon 2 and 5) of *ARL6* gene of selected families RP (04, 06, 21, 23, 43, and 46) would be performed for cross conformation of variants. Next generation sequencing (NGS) is most popular technique now-a-days and it can be used for diagnostic and research purposes. In future complete sequencing of *ARL6* gene should be performed by using NGS platform for identifying the BBS molecular determinants in selected BBS families RP (04, 06, 21, 23, 43, and 46). Genetic counseling was given to all the enrolled and highly inbred families to discourage the cousin marriages in RP and BBS affected families to limit the incidences of disease in future generations. The finding 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 there is a tremendous genetic variations in Bardet-Biedl syndrome. In our study incidence of recessive form of Bardet-Biedl syndrome are high. Different reports from South Asia especially from India and Pakistan are showing *BBS10* and *BBS3/ARL6* as mutational hotspot in Asian population. Due to shortage of budget, in current study only exon 2 and 5 of *BBS3/ARL6* were selected. The results of this study revealed 5 polymorphisms and 4 disease causing mutations in exon 5 of *ARL6* gene out of 5 polymorphisms 4 (g.20299-20300insC, g.20350-20351insCG, g.20388-20389insC, and g.20299-20300insC) were in homozygous condition while one (g.20389-20390insTG) was in heterozygous condition and all were novel while out of four disease causing mutations three (c.266-267insA, c.304-305insGG, c.281T>C) were in homozygous condition while one (266C>A) was in heterozygous condition. Three disease causing mutations (c.266-267insA, c.304-305insGG, and 266C>A) were novel while one (c.281T>C) was previously reported. No genetic variants are observed in exon 2 of ARL6 gene. This study confirms the involvement of *ARL6* gene in BBS syndrome in Pakistan. All BBS families should be initially screened for other variants of *ARL6* gene. Genetic counseling should be given to all the affected families and cousin marriages should be highly discouraged in BBS affected families to limit the incidences of disease in the future generations. .

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