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Genotyping Of Crb1 And Rp1 Genes In Families With Visual Impairment (Retinitis Pigmentosa)

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ABSTRACT

Almost 285 million individuals suffer from vision impairment worldwide in which 90% live in underdeveloped countries. A total of 150 mutations and 60 genes have been associated to Retinitis Pigmentosa. In the current study, two families with Retinitis Pigmentosa were identified in Kohat, Khyber Pakhtunkhwa, Pakistan. The pedigree analysis indicated an autosomal recessive pattern of Retinitis Pigmentosa inheritance. The non-syndromic nature of Retinitis Pigmentosa was confirmed by clinical evaluation of affected members by an eye specialist. Homozygosity mapping was used to analyze the linkage of two families, RP1 and RP2. The microsatellite markers D1S2816, D1S2840, D1S1183, D1S1660, D1S158, D1S422, D1S412, D1S413, D8S532, D8S260, and D8S509 were chosen for

linkage analysis in the RP1 and RP2 families. Affected members of the RP1 family (III.1, III.2, III.3, and III.4) demonstrated homozygosity at the D8S260 marker. Individuals from the RP2 family that were impaired (III.3 and III.4) showed homozygosity at marker D1S1660. The CRB1 locus was used to confirm family linkage in RP1, while the RP1 locus was used to confirm family linkage in RP2. Haplotyping confirmed the allelic pattern in RP1 and RP2 families. Protein-protein interactions were investigated using the Stitch 5 database, and the closest functional partner was discovered. The one closest to CRB1 has a maximum interaction score of 0. 978.The interaction of a mutant protein with other proteins and how it affects the pathway can be predicted by applying this research study. This research could aid in prenatal diagnostics and gene therapy for linked diseases in the future.

Keywords: CRB1, RP1, genotyping, genetic mutation, visual impairment, Retinitis Pigmentosa

1. INTRODUCTION

Traits and molecular inheritance are still important in the twenty-first century, but genetics has progressed beyond inheritance to investigate gene activity, function, distribution, and differences in the context of the cell, organism, and population. Mendelian genetics, molecular genetics, epigenetics, and other subfields of modern genetics have emerged (Griffiths et al., 2000). Scientists can now create new advanced tools to discover and record genetic influences on common and complicated diseases thanks to the completion of the Human Genome Project in 2003. Computational biology, epigenetics, genome-wide association studies, microarray technology, whole-exome sequencing, CRISPR technology, and many other approaches are among the most recent.

1.1. Genetic Disorders

A genetic disorder is an abnormal change within the genome caused by one or more errors. Genetic disorders are caused by changes in the genes or hereditary problems as a result of environmental factors such as smoking, UV exposure, and so on. It can be caused by a mutation in one or multiple genes, or due to a damage in any chromosome. Genetic abnormalities induced by the inheritance of faulty genes from one's parents are referred to as inherited or hereditary disorders. An abnormal chromosomal number causes Huntington's disease, fragile X syndrome, Down syndrome, and other genetic disorders (Stavljenić-Rukavina, 2008).

1.2. Vision Impairment

One of our five senses, vision, allows us to learn a lot about the world around us. The eye is made up of various components that work together and interact with the brain to allow us to see. If any of these elements fail to function or communicate well, vision is hindered. So vision impairment is defined as a considerable functional loss of vision that cannot be corrected by surgical procedures, regular optical lenses such as spectacles, or drugs. Worldwide, it is estimated that 285 million individuals suffer from vision impairment, with 39 million being blind and 246 million having impaired vision (Maberley, 2006). According to WHO (2014), 90% of visually handicapped persons live in underdeveloped countries.

1.2.1. Symptoms

Many signs of vision impairment in children include lack of eye contact, no blinking in strong light, inability to detect moving objects, slow response to various actions, and inability to visually track any moving object placed in front. Symptoms in early childhood include stumbling, glaring, tripping over objects,

depth or color perception, using inappropriate facial expression and body language, gripping objects close to the eyes when looking at them, and so on.

1.2.2. Causes

Inherited eye disorders such as inherited cataract, causes during birth such as retinal difficulties of prematurity, and acquired eye diseases such as corneal infection, tumor of the eyeball, hypoxia, and infection during pregnancy such as rubella infection cause vision impairment (Zundel, 2012). Pakistan is a growing country in South Asia, neighboring China, Iran, Afghanistan, and India. According to Pakistan Bureau of Statistics (2017), with a population of roughly 209,970,000 people, it is the world's fifth most populated country. It consists of these four provinces mainly: Punjab, Khyber Pakhtunkhwa, Sindh, and Baluchistan. Before this survey, Pakistan had just a few research on blindness and visual impairment. Table 1 shows the results of a 2005 study on visual impairment in Pakistan, which looked at a sample of 16507 persons aged 30 and up (Jadoon et al., 2006).

Table 1. Prevalence of Vision Impairment in Pakistan

Province	Normal	Impairment				Total (Age<30)
		Mild Vision	Moderate	Severe	Blind	
Khyber Pakhtunkhwa	2,380	256	342	37	79	3,094
Punjab	6,348	852	1,159	109	336	8,804
Sindh	2,638	315	519	82	110	3,664
Baluchistan	725	68	101	15	36	945
Total	12,091	1,491	2,121	243	561	16,507

1.2.3. Types of Vision Impairment

1.2.3.1. Strabismus: Strabismus is a condition in which the eyes look in multiple directions at the same time and do not focus on a single point. A squint or crossed eyes is another name for it. Strabismus affects 5% of the world's population, the majority of whom are teenagers (Gunton et al., 2015).

1.2.3.2. Congenital Cataracts: According to estimates, 20 million people worldwide suffer from congenital cataracts. Because cataracts cloud the lens of the eye, it diffuses the light that enters the eye and reduces the sharpness of the visual image. Many cataracts develop as a result of eye damage or ageing (Althomali, 2012).

1.2.3.3. Retinopathy of Prematurity: Retinopathy of Prematurity is a condition that affects premature children whose light-sensitive retina has not developed properly before birth. It is estimated that more than 50,000 newborns are affected each year (Gilbert, 2008).

1.2.3.4. Diabetic Retinopathy: Diabetes retinopathy is a serious diabetic eye disease that causes damage to the small arteries and blood vessels above the retina in the back of the eyes. Every year, 8,000 people are affected with diabetic retinopathy (Tapp et al., 2003).

1.2.3.5. Coloboma: A piece of the eye's structure is lost in this disorder. It is an uncommon disorder that affects 0.5 to 0.7 per 10,000 newborns per year (Hornby et al., 2000).

1.2.3.6. Albinism: Albinism is a melanin deficiency that causes a variety of physical issues, including visual impairments. Low vision, such as glare sensitivity and acute light sensitivity, is common in albinos. One out of every 17,000 people is affected by albinism. (Kaplan et al., 2008).

1.2.3.7. Glaucoma: Glaucoma damages the optic nerve by causing fluid buildup and increased pressure inside the eye, resulting in peripheral vision loss and frequent difficulty seeing in dim light. It is estimated that 80 million people are affected globally (Krader et al., 2015).

1.2.3.8. Retinitis Pigmentosa: It is a genetic condition that causes gradual retinal degeneration. It causes severe eyesight loss and retinal degradation. It is a degenerative condition that begins in infancy or adolescence and causes damage to a portion of the visual field as well as impaired night vision (Shintani et al., 2009). Retinitis Pigmentosa is thought to afflict one out of every 3,500 to 4,000 persons (Daiger et al., 2008). Table 2 shows the results of a 2004 study on the prevalence of Retinitis Pigmentosa in Southern Asia. The current research focused on autosomal recessive Retinitis Pigmentosa.

Table 2. Prevalence of Retinitis Pigmentosa in Southern Asia (Source: http://www.rightdiagnosis.com/r/retinitis_pigmentosa/stats-country.html)

Countries	Affected	Population
Afghanistan	10,482	28,513,677
Bangladesh	51,963	141,340,476
Bhutan	803	2,185,569
India	391,570	1,065,070,607
Pakistan	58,528	159,196,336
Sri Lanka	7,318	19,905,165

1.3 Retinitis Pigmentosa (RP)

The most prevalent cause of hereditary visual impairment is represented by RP, globally with a frequency of 1:4,000. RP is a term that describe a set of inherited diseases of retina involving progressive deterioration of rods and cones (light sensing cells), leads to severe visual impairment or blindness. This disease affects the retina or the layer of nerve cells at the posterior of the eye. In the retina there are two types of photoreceptor cells rods and cones cells. Generally, cones cells are present throughout the retina but greatest amount of cones cells are present at center of retina called macula and helps with reading vision and color vision. While rods cells are present at external edge of retina and function in less intense light. In RP photoreceptor cells stop working and result in vision loss (D'Amanda et al., 2020).

Normally RP may be syndromic and non-syndromic. In case of non-syndromic it occurs alone without any other disorders. While in case of syndromic it occurs with any other developmental abnormalities, neurosensory disorders, Secondary to other systemic diseases (Daiger et al., 2013). Projected percentages of RP in autosomal dominant cases is 20-25% while in autosomal recessive cases percentage is 15-20 and

in X-linked cases percentage is 10-15 (Hartong et al., 2006). Around 1 in 4,000 individuals are suffering from non-syndromic form of RP within their lifespan. It is projected that 1.5 million people globally are now affected by RP (Parmeggiani, 2011).

1.4 Sign and Symptoms

Naturally sign and symptoms start in the initially teenage years and in case of severe visual impairment takes place between 40 to 50 years (Kirchner et al., 1987). The clinical hallmarks consist of weakened retinal vessels, an abnormal fundus with bone spicule deposits, abnormal reduced or lacking a- and b-waves in the Electro Retino Gram (ERG) and reduced visual field (Hamel,2006). Early Symptoms that starts in initial teenage years include Blurring of vision, night blindness or nyctalopia followed by reducing visual fields result in tunnel vision and finally legal blindness or in several cases result in complete blindness due to loss of photoreceptor cells (Daiger, 2007).

1.5 Genetics of RP

RP is an inherited eye illness that causes vision loss over time. The retina, which is the light-sensitive tissue layer at the back of the eye, is affected by many eye illnesses. The first sign of RP in children is the loss of night vision (Shintani, 2009). There have been up to 150 mutations associated to RP documented thus far (Daiger, 2013). There are more than sixty genes associated with non-syndromic Retinitis Pigmentosa. Non-syndromic autosomal dominant RP is linked to about twenty-three genes. The non-syndromic autosomal recessive variant of RP has been linked to 55 genes (Rivolta, 2000).

Both non-syndromic autosomal dominant and non-syndromic autosomal recessive RP are linked to about six genes. X-linked RP is linked to six genes (Abigail et al., 2017). Summaries of genes causing non-syndromic RP are shown in Table 3. Mutations in any of the genes connected to RP cause the photo receptor cells (rods and cones) in the retina to gradually deteriorate, resulting in vision loss. Because rod cells naturally die sooner than cone cells, night vision loss is often the first sign of Retinitis Pigmentosa. Late in the day, both rods and cones are destroyed, causing visual problems (Hamel, 2006).

Table 3. Summaries of Genes causing Non-Syndromic RP (Source: http://www.sph.uth.tmc.edu/retnet/)

Disease Category	Total No. of Genes	No. of Identified Genes	Non Identified Genes	Identified Genes
Autosomal Dominant Retinitis Pigmentosa	23	22	RP63	ARL3, ADIPOR1, CA4, CRX, GUCA1B, HK1, FSCN2, TOPORS, SPP2, SNRNP200, SEM4A, RP9, ROM1, RDH12, RPPH2, PRPF31, PRPF8, PRPF6, PRPF4, PRPF3, KLHL7, IMPDH1.

Autosomal Recessive Retinitis Pigmentosa	55	52	RP29, RP32,RP22	ABCA4, ARL6, AGBL5, BBS1, ARL2BP, BBS2, C2orf71, CRB1, C8orf37, CERKL, CLRN1, CNGB1,CNGA1, RLBP1, RGR, RBP3, CYP4V2, DHX38, DHDDS, EMC1,EYS, GPR125, FAM161A, HGSNAT, IDH3B, IFT140, IFT172, IDH3B, KIZ, LRAT, IMPG2, KIAA1549, MAK, MERTK, MVK, SAG, POMGNT1, NEUROD1, PDE6B, PDE6A,PDE6G, PRCD, PROM1, RP1L1, SLC7A14, SPATA7, TRNT1, TTC8, TULP1, ZNF408, USH2A, ZNF513.
X-linked Retinitis Pigmentosa	6	3	RP34, RP24, RP6.	RP2, RPGR, OFD1.
Autosomal Dominant/ Recessive Retinitis Pigmentosa	6	6	0	BEST1, RPE65, RP1, RHO, NRL, NR2E3.

Researchers have identified several different kinds of nonsyndromic Retinitis Pigmentosa, which are generally distinguished based on inheritance patterns such as autosomal dominant, autosomal recessive, and X-linked (Daiger et al. 2007). A single copy of a defective gene is enough to produce disease in an autosomal dominant disorder (Griffiths et al., 2012). In an autosomal dominant disorder, diseased offspring have a negative impact on their parents. In an autosomal recessive inheritance pattern, both parents carry a single copy of the mutated gene but show no signs or symptoms of the disease. Both copies of the gene in each cell must be altered in order for disease to develop (Pelletier et al., 2007).

The genes that cause X-linked disorder are present on the X chromosome in the X-linked inheritance pattern. Because males have two sex chromosomes, one X and one Y, a single faulty copy of the gene in each cell is enough to produce the disease. Because females have two sex chromosomes, both X chromosomes, mutations in both copes of the gene are required to induce illness. However, if a single gene is faulty, at least 20% of females can produce sickness. Males, on average, experience more severe symptoms of illnesses than females. In the case of X-linked inheritance, fathers do not pass on X-linked features to their sons (Sullivan et al., 2006).

1.6 Homozygosity Mapping

Homozygosity Mapping is the mapping of genes in hereditary populations that are linked to rare/recessive disorders. This method takes advantage of the fact that persons with hereditary diseases are more likely to have two copies of the mutant gene from a common ancestor. Because small portions of chromosomal

transfer entirely, sick individuals are most likely to have an identical sequence of alleles at a locus near the illness gene, making them homozygous. Homozygosity maps are used to find genes that are frequently involved in recessive illnesses, as well as to locate homozygosity regions that are shared by dissimilar affected individuals (Sherman, 2008).

1.7 Bioinformatics Analysis

Life science advancements began in the prior 20 years. The advancement of computing and information technology aids in the resolution of a variety of difficulties that arise in daily life. In Bioinformatics, we used computational techniques, information technology, and mathematics to solve problems in biology and medicine. To develop novel biological complications information, it uses signal processing, image processing, artificial intelligence, data mining, modeling, algorithms, and databases (Nair, 2007).

Bioinformatics enables us to evaluate modern biology. Microarray data analysis and BLAST searches for genes and proteins are two major bioinformatics domains (Ungsik et al., 2004). Protein Data Bank and PubMed may contribute newly found data and make adjustments depending on research. (Pearson, 1988; Berman et al., 2000). For example, if we have a specific protein sequence, we can use bioinformatics to compare it to all relevant protein sequences in the Genome data bank. We can use UniProt to locate specific protein sequences in FASTA format and BLAST (Altschul et al., 1997). Because of bioinformatics, there are now 400,000 protein sequences in the protein data bank. We can use computers to retrieve data from all over the world and check 15,000 items in the Protein Data Bank (PDB) (Pearson et al., 1988; Berman et al., 2000 (Pearson et al., 1988; Berman et al., 2000).

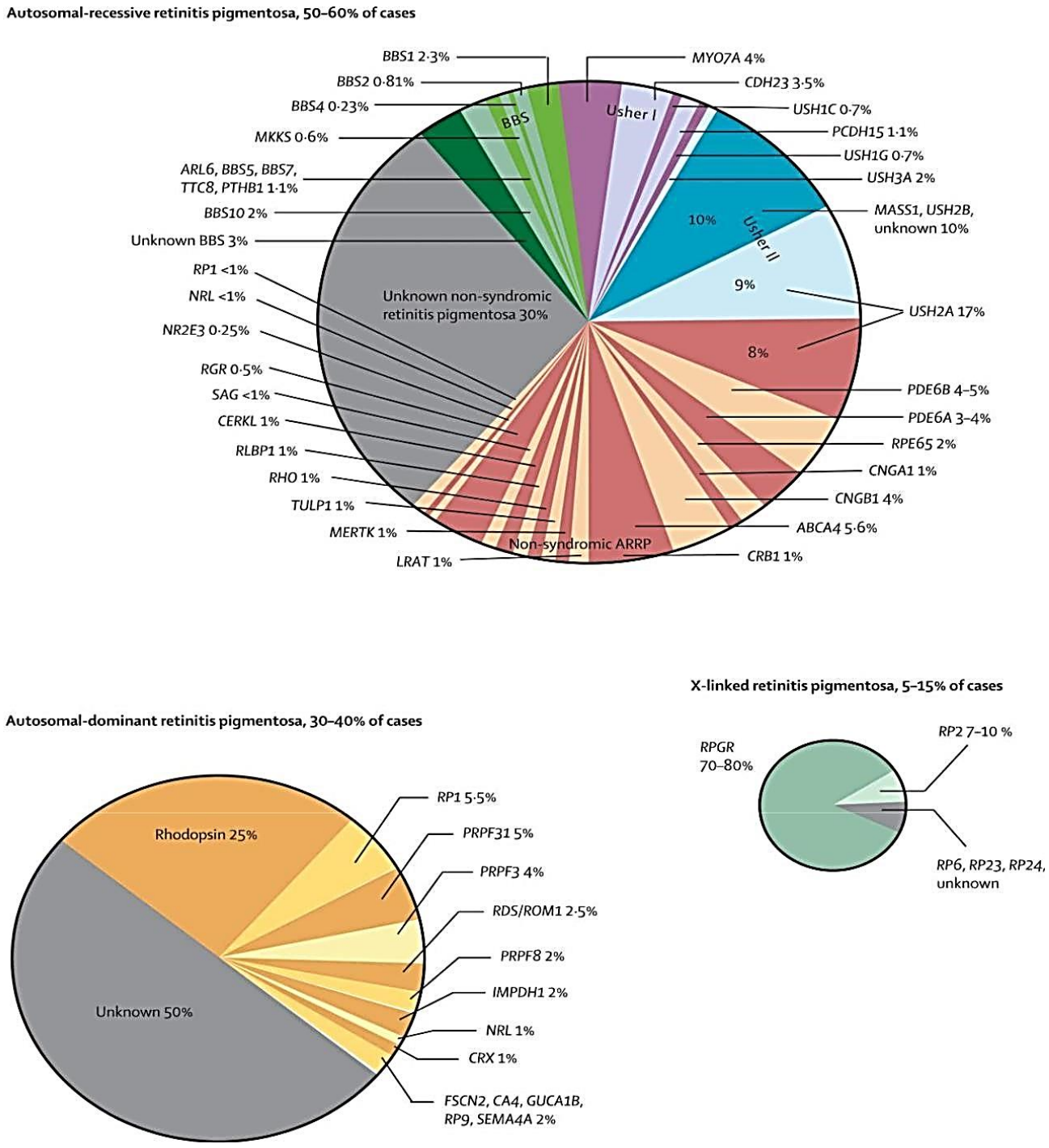


Figure 1. Genes and their role in Non-Syndromic Retinitis Pigmentosa

1.8 Aims and Objectives

In our research study two families showing the clinical phenotypes of RP were examined. Main objectives of research are as follows.

1. Reported loci/genes screening that are involved in the disease in two families.

2. Mutation identification in respective families for possible candidate genes.
3. In silico study of known mutation and their influence on related gene.

2. MATERIALS AND METHODS

2.1. Ascertainment of Families

Two autosomal recessive families with RP were identified in the district of Kohat in Pakistan's Khyber Pakhtunkhwa (KPK) region. On the basis of private interaction, these two RP1 and RP2 families were chosen. The disease history and inheritance pattern were determined by paying a visit to their place of residence. The families of the participants gave their informed consent to participate in the study. The impacted member's medical records were checked, and the findings were noted. An eye specialist examined both normal and affected members of this family, and diseased related features were documented.

2.2. Pedigree and Clinical Assessment

Each family's pedigree was created using the traditional procedure. Pedigree analysis is a methodology for displaying family data and analyzing genetic inference. It gives details on how an inherited disorder is passed down across the generations within a family. In the pedigree, a variety of statistical signs were applied. Unfilled squares represent normal males, whereas unfilled circles represent normal females. The male and female victims are depicted by a filled square and a circle, respectively. The consanguineous link between the families is indicated by the double line in the pedigree. Families' deceased members were depicted by an oblique line on a square or circle. Generations are signified by Arabic numerals, whereas individuals are denoted by Roman numerals. The number of siblings is indicated by the enclosed numbers within a symbol. An eye specialist conducted a clinical evaluation as well.

2.3. Collection of Blood in EDTA Tubes

Blood was extracted both from the impaired and unaffected members of each family using 10mL sterilized syringes (0.7 x 40mm 0.22 x 112; (BD, USA), while blood was extracted from butterflies from those under the age of two years. After that, the syringes were emptied into a 10mL EDTA-filled tube and kept at 4°C in the lab.

2.4. Genomic DNA isolation from samples

Organic method was used for the isolation of genomic DNA from family members.

2.4.1. Genomic DNA isolation using Standard Phenol-Chloroform Method

Protocol used for preparing genomic DNA is as under:

- In a 1500µL centrifuge tube, 0.5mL blood was taken and mixed with an equal amount of solution A. After leaving the tubes at room temperature for 15-20 minutes, shake the Eppendorf tubes and move them up and down to mix the solution A with the blood. In a table top mini centrifuge, the tubes were centrifuged at 13000rpm for 10-12 minutes (Model 5415D, Eppendorf, Germany).

- The supernatant was removed after centrifugation, and the remaining pellet was re-suspended in 400µL of solution A. After vortexing the tube and mix the pallet in solution A, centrifuge it at 13000rpm for 1.12 minutes.
- Carefully removed the supernatant. After removing the supernatant, add around 400µL of Solution B, 25µL of proteinase –K (25 mg/mL), and 12µL of 20 percent sodium dodecyl sulphate (SDS). The Eppendorf tube is spun for 15 seconds to mix the solutions and pallet. The tubes were then transferred in the incubator and kept at 37°C overnight.
- The next day, 250µL of solution C and 250µL of solution D were added to an Eppendorf tube and thoroughly mixed before centrifuging for 1.12 minutes at 13000rpm. After centrifugation, around 600 µL of supernatant was collected in a new Eppendorf tube.
- After adding an equivalent volume of solution C and gently shaking the tube, the tubes were left for 5-10 minutes. Eppendorf tube centrifuged for 1.12 minutes at 13000rpm.
- After centrifugation, remove around 800µL of the supernatant in a separate Eppendorf tube, add 700µL cold iso-propanol, and leave tubes for 5-10 minutes to precipitate the DNA at the bottom. At 13000rpm, an Eppendorf tube was centrifuged for 1.12 minutes.
- To eliminate contaminants from DNA, 10µL of 100% ethanol are applied. To dissolve the DNA, 100µL T.E buffer was added and kept at 4 ° c overnight in the refrigerator.

The following solutions were used for DNA extraction

- Solution A: 0.32 M sucrose, 10mM Tris pH 7.5, 5mM MgCl2, 1% Triton X-100.
- Solution B: 10mM Tris pH 7.5, 400mM NaCl, 2mM EDTA pH 8.0.
- Solution C: Buffered Phenol.
- Solution D: Chloroform (24mL), Isoamyl Alcohol (1mL).

2.5. Horizontal Gel Electrophoresis

Isolated DNA samples were analyzed on a 1% agarose gel, which was manufactured by dissolving 0.5g of agarose in 50mL of 1X TBE (89 mM Tris-Borate, 2.5 mM EDTA, pH 8.3) and melting it for one minute in a microwave oven. Following permitting the solution to cool, add 2µL of ethidium bromide solution (0.1g/mL final concentrations) to enable for DNA visibility after electrophoresis. The prepared mixture was then poured in the gel caster and left for 30-40 minutes to solidify. Amplified PCR products were examined on a 2% agarose gel, which was synthesized by dissolving 0.8g of agarose in 40mL of 1X while following the same procedure as the last one.

The amplified DNA and PCR products samples were combined with 2µl and 3µl Bromophenol Blue dye (0.25 percent bromophenol blue with 40 percent sucrose solution) after loading the gel to the gel tank. After injecting the samples into the gel's wells, electrophoresis was carried out at 120 volts (400 mA) for 20 minutes in 1X TBE buffer. After the electrophoresis gel was ready, it was analyzed in Dolphin-Doc (WEALTEC, USA 2010-2011).

2.6. Primer Designing

Microsatellite markers were examined for known loci associated with PR syndrome in attempt to set up linkage. Primer samples were extracted from the Rat Genome Database website, and a list of microsatellite markers applied in linkage analysis of known RP loci in families is provided in table 4.

2.7. Polymerase Chain Reaction (PCR)

The polymerase chain reaction was performed in 200 mL tubes with a total reaction volume of 20µl. The reaction mixture was created by combining the following ingredients:

- 9.6µl PCR water
- 2µl 10 X buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl)
- 2µl MgCl2 (25 mM)
- 0.2µl dNTPs (10 mM)
- 2µl sample DNA (40ng)
- 2µl of each forward and reverse primer (0.1µM)
- 0.2µl Taq DNA polymerase (5 unit/µl, Fermentas, USA)

The prepared reaction mixture was vortexed for a few seconds. The reaction mixture was ready for Thermocycling conditions, that included the stages below: The first step is DNA denaturation, which requires heating DNA double strands to 94°C for 30 seconds to convert them to single strands: The second step is annealing, in which primers "anneal" to their complementary sequences on either side of the target sequence at a low temperature of 45 - 62°C for 1 minute (depending on the Tm of primers): the third and final step is extension or elongation, in which complementary DNA strands from each primer are elongated for one minute at 72°C, with the final 10 minutes at 72°C for Taq DNA polymerase to synthesized.

2.8. Vertical Gel Electrophoresis

To detect amplified PCR output, polyacrylamide gels are utilized. Two thin glassware plates were needed in this method, which were clipped together and spaced by 1.5 mm. A 250 mL beaker was filled with an 8% non-denaturing polyacrylamide gel mixture, which was then sandwiched thin glassware plates. The plates containing the gel mixture were allowed to solidify for 45-60 minutes at room temperature after the comb was attached. The gel glasses plates were settled into the vertical gel tank after polymerization, and amplified DNA samples were combined with 5µl of loading dye (0.25 percent bromophenol blue with 40% sucrose) and put into the wells. The vertical gel tank type V16-2 (Life Technologies, USA) was used for electrophoresis at 130 volts (60 mA) for 90-100 minutes, depending on the size of the amplified length. Gel Doc system was used to observe the gel, which was stained with ethidium bromide solution (10 mg/mL) (BioRad, Italy).

Table 4. List of Microsatellite Markers used for Linkage Analysis

Gene	Primers	Sequence
CRB1	D1S2816	**Forward Primer** TTCCCCAAATGTATTACTGC **Reverse Primer** AAAGGAGTACCCAATCCCAG

	D1S2840	**Forward Primer** GACAAGTCATCTTACACCTCAGTTC **Reverse Primer** CCAACATAATTTCTGGGCTG
	D1S1183	**Forward Primer** TCTTCATTTTTTTCTCTCCTC **Reverse Primer** ACAAACTCTGAAGCTGAAGA
	D1S1660	**Forward Primer** TGCTATCCTCTCACCAGTGA **Reverse Primer** GTCTGAAGTTCATGGGAACG
	D1S158	**Forward Primer** GGGCCTTCTTATATTGCTTC **Reverse Primer** GGAAAGACTGGACCAAAGAG
	D1S422	**Forward Primer** TTCCACAGTCATTTGAGTCC **Reverse Primer** TCTCTAGAGAAGCAGAGCCA
RP1	D8S532	**Forward Primer** GCTCAAAGCCTCCAATGAC **Reverse Primer** GACTTCGTGATCCACCTGC
	D8S260	**Forward Primer** AAATGTGAGGCTGTTGGCTTTAC **Reverse Primer** TTGTCTGCTGAAGGCTGTTCTATG
	D8S509	**Forward Primer** ACGTTTACCTGTGTAACAAACC **Reverse Primer** AATGGTACCAGCACACGTAG
	D8S1737	**Forward Primer** TGTAACACACAGATGCACGC **Reverse Primer** AAGGATAACTTTGGCTTCGG
	D8S2332	**Forward Primer** ATACAGGCTCGTTGCTGAAG **Reverse Primer** CCATTGCAAAGCAGTCTTCT

Table 5. Composition of 8% Polyacrylamide Gel

S.No.	Chemicals	Amount
1	Distilled water	31.13 mL
2	10 X TBE	5 mL
3	30% Acrylamide solution (29g polyacrylamide, 1g Methylene-bisacrylamide)	13.5 mL
4	10% Ammonium persulphate (APS)	350 µL
5	TEMED (N, N, N', N'-Tetra Methyl Ethylene Diamine)	20 µL

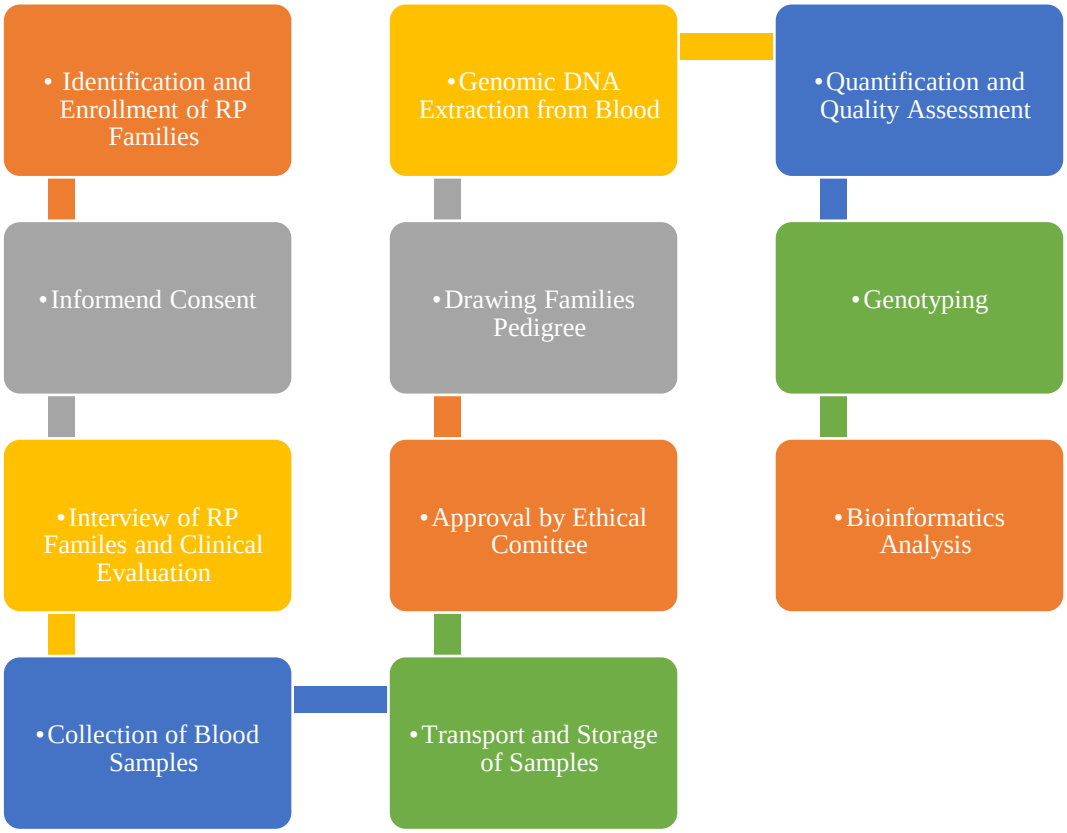


Figure 2. Flow chart showing the summary of current study

3. RESULTS

3.1. Families Description and Assessment

Current study used for research includes two RP families labelled as RP1 and RP2 and affected members of both the families show symptoms of Retinitis Pigmentosa. Description of the both RP1 and RP2 families are given below.

3.1.1. Family RP1

Family RP1 residing in district Kohat in Khyber Pakhtunkhwa region of Pakistan. The pedigree showed autosomal recessive mode of inheritance. The family consists of three generations having normal parents (II.8 and II.9) with one normal female (III.5) and 4 affected males (III.1, III.2, III.3 and III.4) as displayed in the pedigree (Figure 2). Affected family members neither showed any additional symptoms, such as speech disorder, deafness etc. clinical examination of all affected members show same phenotype (Table 6). Pedigree was constructed using Cyrillic software (Figure 2) and consent form was signed before collection of blood samples. Peripheral blood samples were taken from seven individuals, four affected brothers (III.1, III.2, III.3 and III.4), normal father and mother (II.8 and II.9) and one normal sister (III.5).

3.1.2. Family RP2

Family RP2 residing in district Kohat in Khyber Pakhtunkhwa region of Pakistan. The pedigree showed autosomal recessive mode of inheritance. The family consists of three generations having normal parents (II.1 and II.2) with one normal male and one normal female (III.1 and III.2) and two affected females (III.3 and III.4) as displayed in the pedigree (Figure 4). Affected family members neither showed any additional symptoms, such as speech disorder, deafness etc. clinical examination of all affected members show same phenotype (Table 7).

Pedigree was constructed using Cyrillic software (Figure 3) and consent form was signed before collection of blood samples. Peripheral blood samples were taken from five individuals, two affected sisters (III.3 and III.4), one normal brother and sister (III.1, III.2) and normal mother (II.1), Father (II-2) was not available at the time of sampling.

3.2. Linkage Analysis through Homozygosity/Auto zygosity Mapping

Microsatellite markers were used for the purpose of Auto zygosity mapping and genotyped for normal and affected members of the respective families were created and given below under each family.

3.2.1. Family RP1

In family RP1 seven DNA samples (II.8, II.9, III.5, III.1, III.2, III.3 and III.4) including three normal and four affected members were genotyped. In this family linkage was established with microsatellite marker D8S260 at CRB1locus (Figure 8). Heterozygous patterns were showed by affected individuals when used microsatellite markers D1S1660, D1S2816 and D8S532 (Figure 5, 6 and 7). By haplotyping the family allelic patterns were determined (Figure 12).

3.2.2. Family RP2

In family RP2 five DNA samples (II.1, III.1, III.2, III.3 and III.4) including two affected and three normal individuals were genotyped except from II.2 individual. In this family linkage was established with microsatellite marker D1S1660 at RP1 locus (Figure 11). Heterozygous pattern was showed by affected individuals when used microsatellite markers D1S2816 and D1S1723 (Figure 9 and 10). By haplotyping the family allelic patterns were determined (Figure 13).

3.3. Bioinformatic Analysis

Protein proteins interaction was examined and nearest relative efficient partner was recognized through Stitch 5 database. The nearest one with maximum interaction score of 0.978 with CRB1was nominated for additional examination and consider as protein ligand i.e. MPP5 (Figure 16). Membrane Palmitoylated Protein 5 play important role in establishment of cell polarity in epithelial cells and may play a role in the dynamic remodeling of the apical cytoskeleton. The nearest one with maximum interaction score of 0.862 with RP1 was nominated for additional examination and consider as protein ligand i.e. MAPRE2 (Figure 17). Microtubule Associated Protein RP/EB Family Member 2 may be involved in microtubule polymerization, and spindle function by stabilizing microtubules and role in migration

(http://stitch.embl.de/). Expression of CRB1and RP1 in different organs of human body was analyzed through UCSC genome browser (Figure 14 and 15) (https://genome.ucsc.edu).

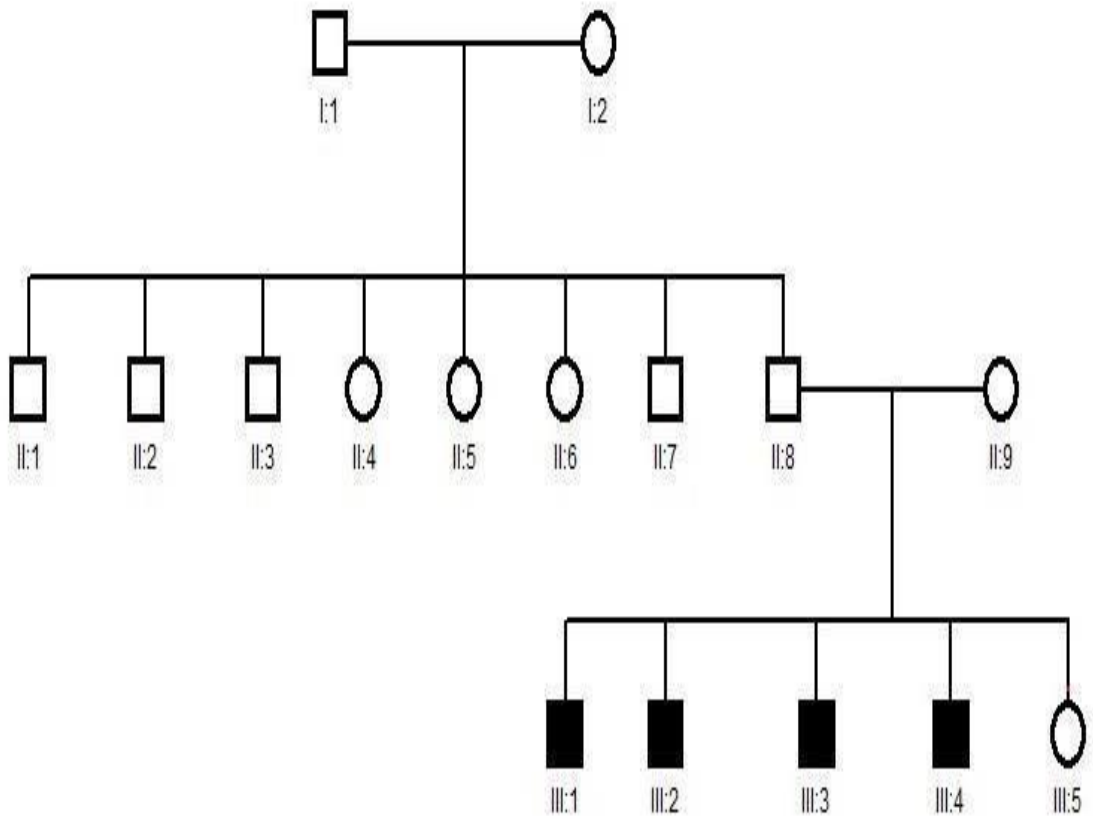


Figure 3: Pedigree of family RP1. Square shows male individual although circles represent the female individuals. Filled squares shows affected male and filled circle shows affected female.

Table 6. Clinical features of Family RP1

Clinical Features	**II.8**	**II.9**	**III.5**	**III.1**	**III.2**	**III.3**	**III.4**
Sex	Male	Female	Female	Male	Male	Male	Male
Phenotype	Normal	Normal	Normal	Affected	Affected	Affected	Affected
Age	43	38 years	20 years	18 years	16 years	13 years	11 years

Speech Disorders	-	-	-	-	-	-	-
Deafness	-	-	-	-	-	-	-
Schooling	-	-	+	-	-	-	-
Night Blindness	-	-	-	-	-	-	-
Eye Brows	Systematically Aligned	+	+	+	+	+	+
Lacrimal Gland	No Tearing	No Tearing	No Tearing	No Tearing	No Tearing	No Tearing	No Tearing
Sclera	White	White	White	White	White	White	White
Iris	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Pupils	Black	Black	Black	Black	Black	Black	Black
Blurry Vision	No	No	No	No	No	No	No

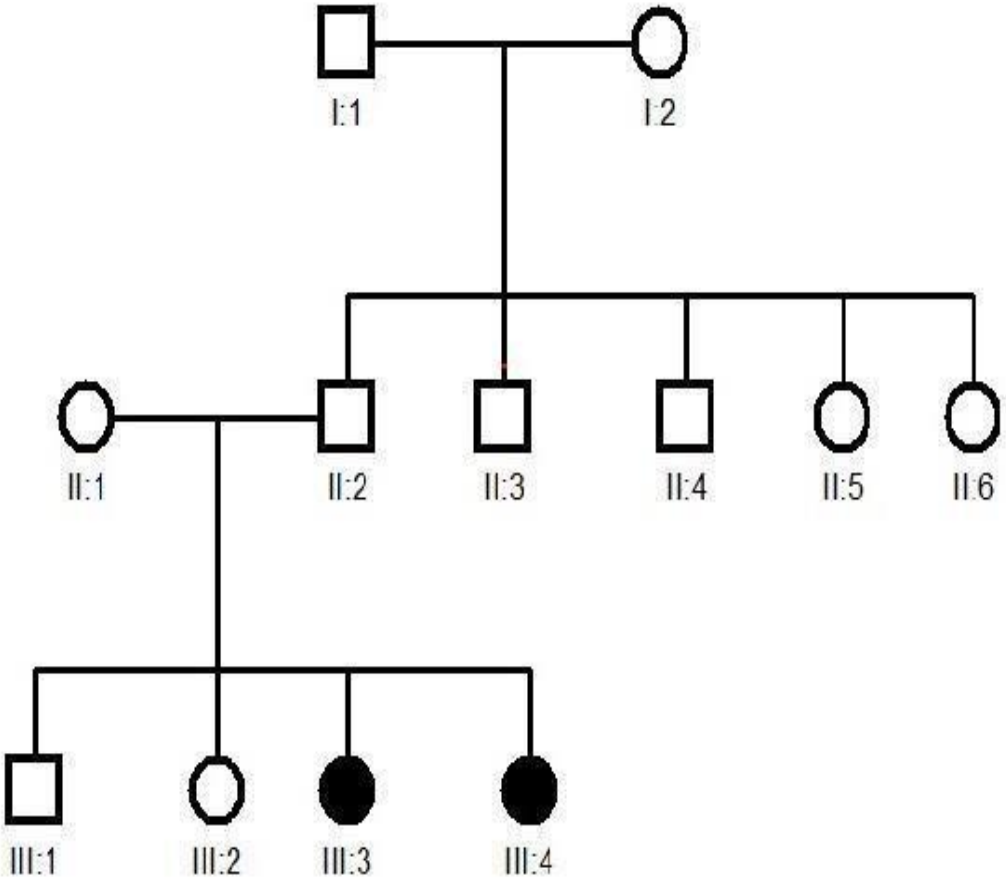


Figure 4: Pedigree of family RP2. Square shows male individual although circles represent the female individuals. Filled squares shows affected male and filled circle shows affected female

Table 7. Clinical features Family RP2

Clinical Features	II.1	II.2	III.1	III.2	III.3	III.4
Sex	Female	Nil	Male	Female	Female	Female
Phenotype	Normal	Nil	Normal	Normal	Affected	Affected
Age	40 years	Nil	12 years	17 years	19 years	15 years
Speech Disorders	-	Nil	-	-	-	-

Deafness	-	Nil	-	-	-	-
Growth	Normal	Nil	Normal	Normal	Normal	Normal
Schooling	-	Nil	+	+	-	-
Night Blindness	-	Nil	-	-	-	-
Eye Brows	Systematically Aligned	Nil	+	+	+	+
Lacrimal Gland	No Tearing	Nil	No Tearing	No Tearing	No Tearing	No Tearing
Sclera	White	Nil	White	White	White	White
Iris	Yes	Nil	Yes	Yes	Yes	Yes
Pupils	Black	Nil	Black	Black	Black	Black
Blurry Vision	Nil	No	No	No	No	No

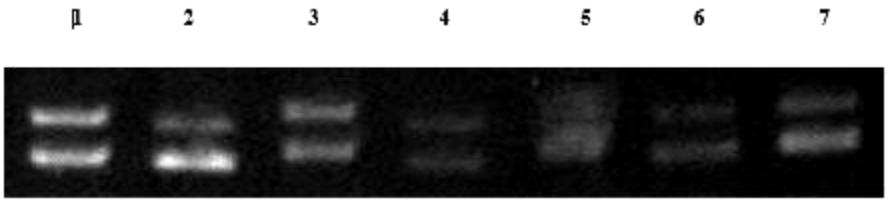


Figure 5: Electropherogram of marker D1S1660, ethidium bromide stained with 8% non-denaturing polyacrylamide gel. The Arabic and roman digits represent family members in pedigree. (1. II.8 Normal 3. III.5 Normal 5. III.2 Affected 7. III.4 Affected 2. II.9 Normal 4. III.1 Affected 6. III.3 Affected)

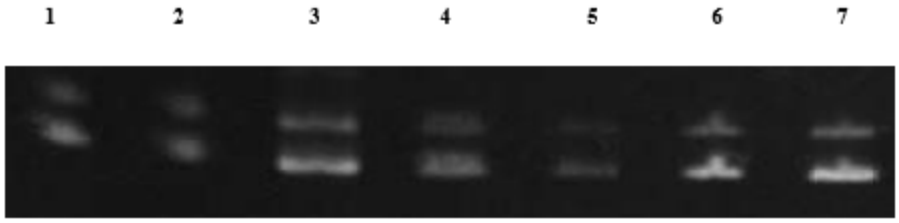


Figure 6: Electropherogram of marker D1S2816, ethidium bromide stained with 8% nondenaturing polyacrylamide gel. The Arabic and roman digits represent family members in pedigree. (1. II.8 Normal 3. III.5 Normal 5. III.2 Affected 7. III.4 Affected 2. II.9 Normal 4. III.1 Affected 6. III.3 Affected)

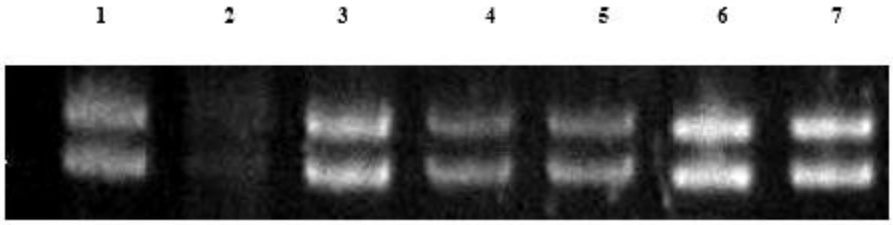


Figure 7: Electropherogram of marker D8S532, ethidium bromide stained with 8% nondenaturing polyacrylamide gel. The Arabic and roman digits represent family members in pedigree. (1. II.8 Normal 3. III.5 Normal 5. III.2 Affected 7. III.4 Affected 2. II.9 Normal 4. III.1 Affected 6. III.3 Affected)

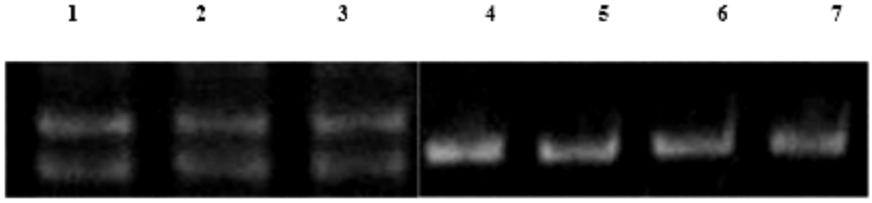


Figure 8: Electropherogram of marker D8S260, ethidium bromide stained with 8% nondenaturing polyacrylamide gel. The Arabic and roman digits represent family members in pedigree. (1. II.8 Normal 3. III.5 Normal 5. III.2 Affected 7. III.4 Affected; 2. II.9 Normal 4. III.1 Affected 6. III.3 Affected)

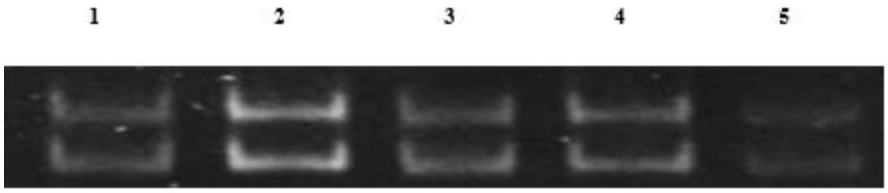


Figure 9: Electropherogram of marker D1S2816, ethidium bromide stained with 8% nondenaturing polyacrylamide gel. The Arabic and roman digits represent family members in pedigree. (1. II.1 Normal 3. III.2 Normal 5. III.4 Affected; 2. III.1 Normal 4. III.3 Affected)

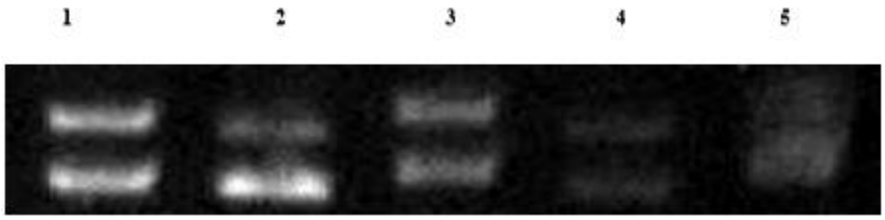


Figure 10: Electropherogram of marker D1S1723, ethidium bromide stained with 8% nondenaturing polyacrylamide gel. The Arabic and roman digits represent family members in pedigree. (1. II.1 Normal 3. III.2 Normal 5. III.4 Affected. 2. III.1 Normal 4. III.3 Affected)

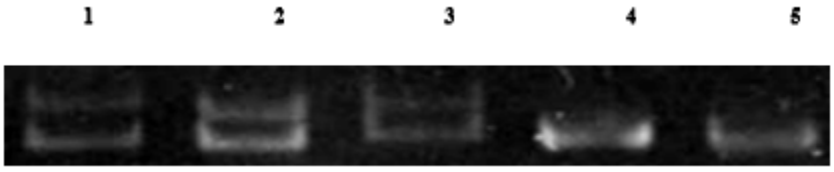


Figure 11: Electropherogram of marker D1S1660, ethidium bromide stained with 8% nondenaturing polyacrylamide gel. The Arabic and roman digits represent family members in pedigree. (1. II.1 Normal 3. III.2 Normal 5. III.4 Affected 2. III.1 Normal 4. III.3 Affected)

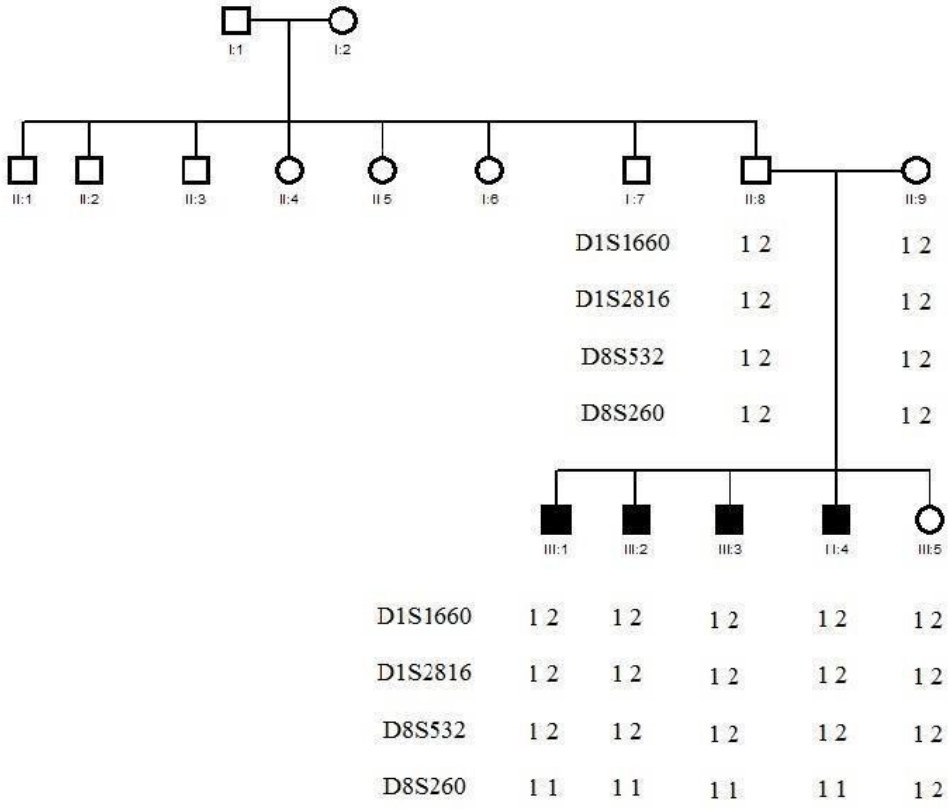


Figure 12: Haplotype of RP1 Family Displaying Allelic Pattern and showing linkage interval at CRB1 locus.

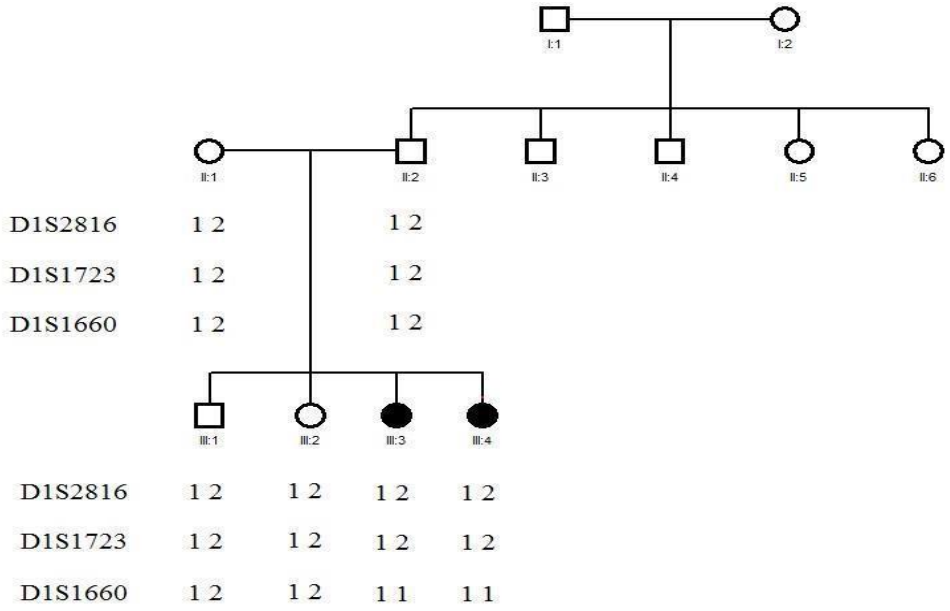


Figure 13: Haplotype of RP2 Family Display Allele Pattern and showing linkage interval at RP1 locus.

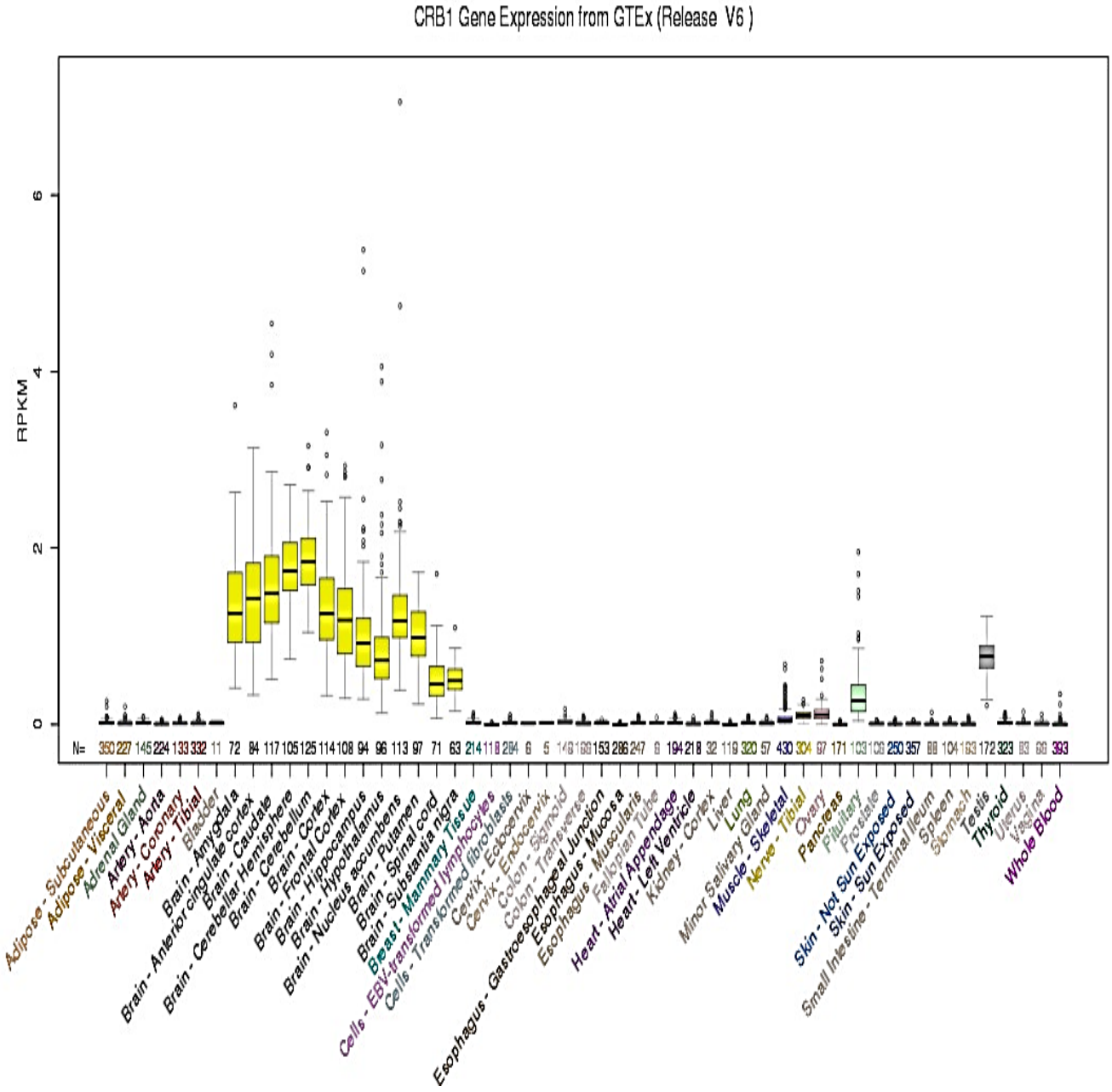


Figure 14: Expression of CRB1Gene in Different Organs of Human Body

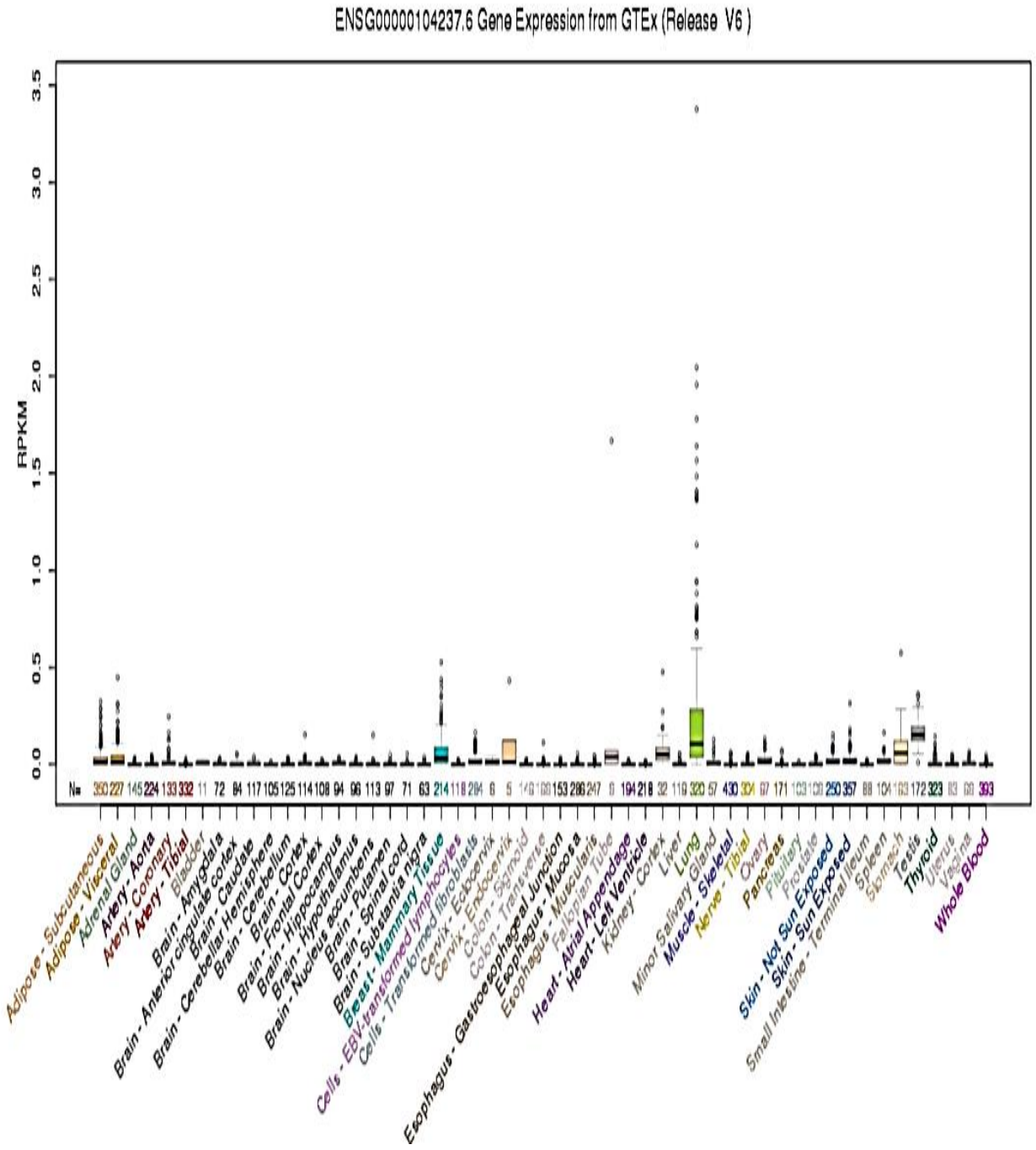


Figure 15: Expression of RP1 Gene in Different Organs of Human Body

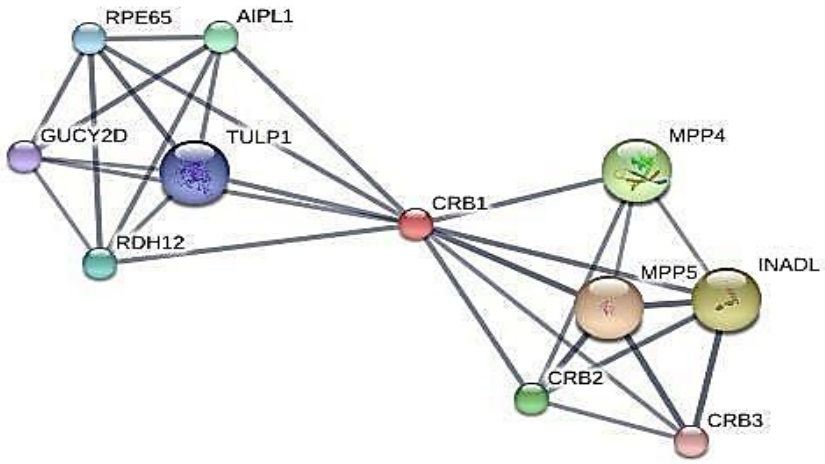


Figure 16: STITCH5 Results. Pictorial Representation of Interaction of CRB1with its Possible Ligands. Denser lines represent stronger links. Protein-protein interactions are displayed in blue, chemical-protein interactions are shown in green and chemical-chemical interaction shown in red.

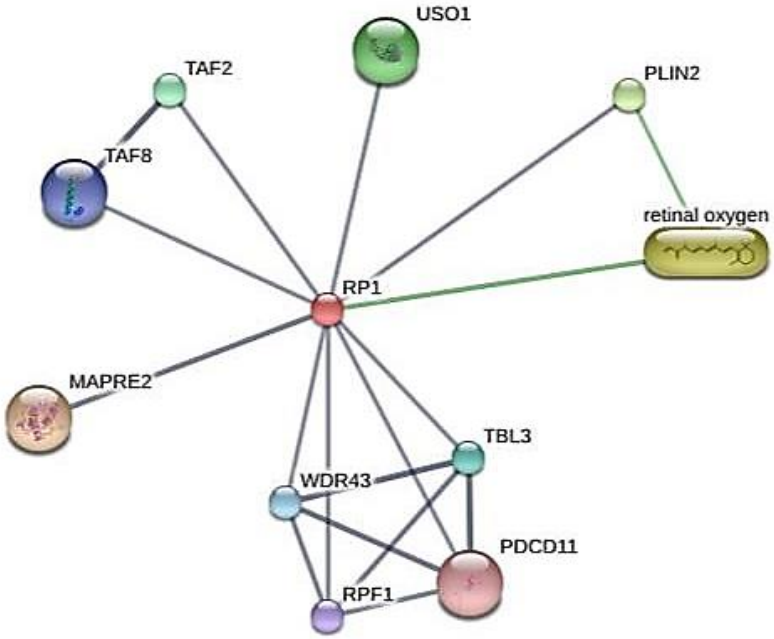


Figure 17: STITCH5 Results. Pictorial Representation of Interaction f RP1 with its Possible Ligands. Denser lines represent stronger links. Protein-protein interactions are displayed in blue, chemical-protein interactions are shown in green and chemical-chemical interaction shown in red.

Discussion

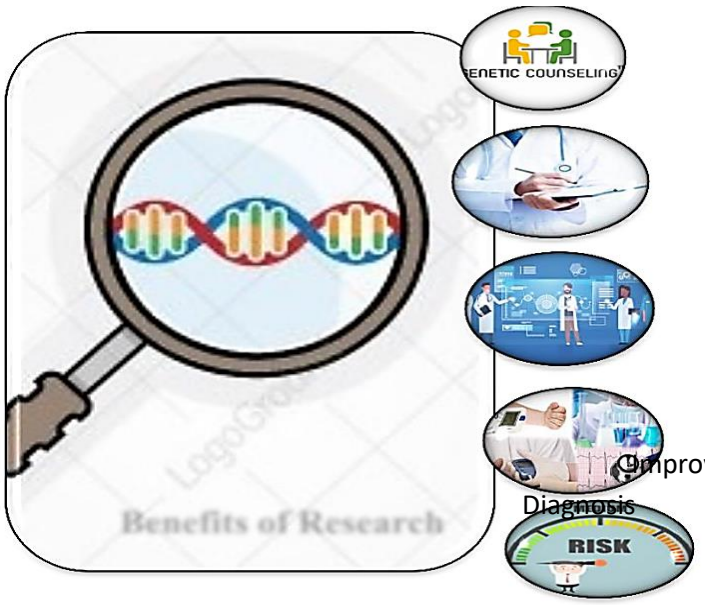
Autosomal recessive RP is caused due to the mutation in fifty-five genes and out of fifty-five genes fifty-two genes are known. Mutation in CRB1 and RP1 are the most common causes of autosomal recessive RP in in Khyber Pakhtunkhwa of Pakistan. CRB1 is the gene present on chromosome number 1 having 18 exons and it encode protein called Crumbs homolog 1. This protein is found in the brain and in the retina

that detects light and color. RP1 gene is present on chromosome number 8 having 10 exons and it encodes protein called RP 1 protein. RP1 protein play role in maintenance and tissue growth. Two families RP1 and RP2 suffering from RP residing in district Kohat Khyber Pakhtunkhwa region of Pakistan was ascertained for current study and Pedigree analysis revealed the autosomal recessive pattern of inheritance of RP in these two families. PR1 family has normal parents (II.8 and II.9) with one normal female (III.5) and 4 affected males (III.1 III.2 III.3 III.4). PR2 family has normal parents (II.1 and II.2) with one normal male and one normal female (III.1 and III.2) and two affected females (III.3 III.4). Clinical examination of affected member of these two families by eye specialist confirmed non syndromic nature of retinitis pigmentosa. Linkage analysis of two families RP1 and RP2 was performed in this study through homozygosity mapping. In linkage analysis microsatellite markers were used for mapping disease associated loci in this study. In family RP1 and RP2 microsatellite markers D1S2816, D1S2840, D1S1183, D1S1660, D1S158, D1S422, D1S412, D1S413, D8S532, D8S260 and D8S509 were used for linkage study. Family RP1 showed linkage with markers D8S260 as affected individuals (III.1, III.2, III.3 and III.4) shown homozygosity at marker D8S260. While RP2 family showed linkage with markers D1S1660 as affected individuals (III.3 and III.4) shown homozygosity at marker D1S1660. In RP1 family linkage was confirmed in CRB1 gene while in RP2 family linkage was confirmed in RP1 gene. By haplotyping the family RP1 and family RP2 allelic pattern was determined Protein proteins interaction was examined and nearest relative efficient partner was recognized through Stitch 5 database. The nearest one with maximum interaction score of 0.978 with CRB1 was nominated for additional examination and consider as protein ligand i.e. MPP5. The nearest one with maximum interaction score of 0.862 with RP1 was nominated for additional examination and consider as protein ligand i.e. MAPRE2. Further mutation identification in the desired families was to be confirmed through Sanger's Sequencing. By using this research study, it can be predicted the interaction of mutated protein with other protein and how it is affecting the pathway and resulting into a disorder. This study can help in future in prenatal diagnosis and gene therapy to related disease.

4. Conclusion

Linkage analysis of two families RP1 and RP2 was performed in this study through homozygosity mapping. In linkage analysis microsatellite markers were used for mapping disease associated loci in this study. In family RP1 and RP2 microsatellite markers D1S2816, D1S2840, D1S1183, D1S1660, D1S158, D1S422, D1S412, D1S413, D8S532, D8S260 and D8S509 were used for linkage study. Family RP1 showed linkage with markers D8S260 as affected individuals (III.1, III.2, III.3 and III.4) shown homozygosity at marker D8S260. While RP2 family showed linkage with markers D1S1660 as affected individuals (III.3 and III.4) shown homozygosity at marker D1S1660. In RP1 family linkage was confirmed in CRB1gene while in RP2 family linkage was confirmed in RP1 gene. By haplotyping the family RP1 and family RP2 allelic pattern was determined

Protein proteins interaction was examined and nearest relative efficient partner was recognized through Stitch 5 database. The nearest one with maximum interaction score of 0.978 with CRB1was nominated for additional examination and consider as protein ligand i.e. MPP5. The nearest one with maximum interaction score of 0.862 with RP1 was nominated for additional examination and consider as protein ligand i.e. MAPRE2.



Genetic Counseling

Improve understanding of molecular basis of inherited disorders

Health Care Development

Improve methods for Disease Diagnosis

To help educate other family members about potential risk

Figure 18: Shows research outcomes

Future perspective

Sanger's Sequencing should be used to confirm further mutation discovery in the desired families. The interaction of a mutant protein with another protein can be predicted utilizing this research study. These findings should be incorporated in future studies related to the altered route that leads in a problem in order to aid in prenatal diagnostics and gene therapy for associated diseases.

Conflict of Interest: The authors declare that they have no conflict of interest.

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Author Contributions: Syed Anees Ali, Muhammad Sajid and Arsalan Rasheed designed the work; Muhammad Hashim Raza collected the data; Hamid Mehmood and Muhammad Sajid performed the Lab work; Bakht Beland and Ma. Socorro Gonzaga-Leong-on drafted the manuscript; Tania Naveel and Maria Binte Sarfraz performed the analysis and Tanmay Ghosh proof read and revised the manuscript for correction and incorporation of required changes. All authors reviewed the manuscript and approved the final version for submission.

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