Genotype-Phenotype Correlation for families affected with Oculocutaneous Albinism

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It is certified that we have evaluated the thesis entitled "***Genotype-Phenotype Correlation for Families Affected with Oculocutaneous Albinism***" submitted by Jabbina Fawwad (627-FOS/MSBT/F23), and it is our judgment at this project is of sufficient standard to warrant its acceptance by the International Islamic University Islamabad for the MS degree in Biotechnology.

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DEDICATION

"In the name of Allah, the Most Gracious, the Most Merciful"

This thesis is dedicated to the One and Only God, who gave me the strength and guidance to complete this work. I am grateful for the blessings and opportunities He has bestowed upon me throughout my academic journey.

This thesis is dedicated to my Late Grandfather, **Prof. Abdul Qayyum Shakir**, who is my inspiration.

This thesis is dedicated to my loving and supportive Father, **Muhammad Fawwad Shakir**, and my beloved mother, **Rubina Rehman**, who have always supported me throughout my academic journey with love, encouragement, and sacrifice. Without their support, I would not have been able to reach this milestone.

Last but not least, I dedicate this thesis to my beloved sisters and best friends, Aqsa and Iqra, who has always been there for me. Thank you for your support, motivation, and love. This achievement would not have been possible without you.

Lastly, I dedicate this thesis to my loving younger sisters, Arzoo and Pakeeza, and my Lab Colleagues.

DECLARATION

I, Jabbina Fawwad, declare that this thesis entitled "Genotype-Phenotype Correlation for families affectedwith Oculocutaneous Albinism" is the result of our original research and has not been previously submitted for any academic qualification.

Dated: _____

JabbinaFawwad

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Abstract

A genetically diverse spectrum of conditions known as oculocutaneous albinism is typified by decreased or nonexistent melanin synthesis, which results in hypopigmentation of the skin, eyes, or hair. With an emphasis on identifying pathogenic variations which lead to the condition, this study's main goal was to examine the relationship between genotype and phenotype in OCA-affected Pakistani households. To find the cause of the mutations, two closely related consanguineous families from Khyber Pakhtunkhwa were enlisted, and thorough clinical assessments and whole exome sequencing (WES) were carried out.

In both families, an entire set of four OCA2 gene variations were found. Three previously documented variants were found in one family (IIU-OCA-10): two missense variants (c.2158C>T; p.Arg720Cys and c.1211C>T; p.Thr404Met) that occurred in a compound heterozygous form, and one synonymous variant (c.1776G>A; p.Ala592=). It was anticipated that these missense changes would be harmful and linked to a more severe phenotype. Anintronic variant (c.1045-15T>G) was found in the second family (IIU-OCA-08) which may alter splice site or exon skipping, it can be assess by splice prediction tools or minigene assay. Several in silico methods predicted that this variant would be harmful, yet it is not included in any public databases.

From a clinical perspective every affected person had common signs of OCA, such as pale skin color, photophobia, nystagmus, white to reddish-white hair, and impaired visual acuity. The diagnosis was confirmed by direct ophthalmoscopic examination, which showed foveal hypoplasia and a hypopigmented albinotic fundus. A strong genotype-phenotype link is supported by the prevalence of these mutations and consistent symptoms, which further identify OCA2 as a significant contributor to OCA in the Pakistani community.

This study broadens the mutational spectrum of OCA2 and contributes to the increasing amount of evidence about the genetic basis of OCA. The identification of a novel variant highlights the significance of carrying out genetic research relevant to a population, particularly in areas where consanguineous marriages are common. The development of future gene-targeted treatments for OCA in Pakistan, clinical diagnosis, and genetic counseling are all significantly impacted by these findings.

CHAPTER NO: 01
INTRODUCTION

Introduction

Oculocutaneous Albinism is a hereditary disorder that is autosomal recessive. The condition is autosomal recessive when both parents are heterozygous or carriers of it. Children of carrier parents have a 25% chance of inheriting the disease. Oculocutaneous Albinism is characterised by hypopigmentation of the skin, hair, and eyes due to either a decrease in melanin production or a complete absence of it in the melanocytes (Grønskov, 2007). These disorders generally come on by mutations in certain genes that are required for melanocytes, specialised cells, to produce melanin pigment. Melanin pigment comes in two different forms: yellow-red pheomelanin and brown-black eumelanin (Yan, 2005). In the skin and hair, melanocytes transfer melanin to keratinocytes, while the iris and retinal pigment epithelium of the eye create pigment. Pale skin, which is very susceptible to UV damage and can result in skin cancer known as melanoma, and abnormal eye development that impairs vision are caused by insufficient or missing melanin spigment. Among the defects of the vision are photophobia, nystagmus, and strabismus. Misrouting of the optic nerves and foveal hypoplasia are other alterations (Dorothy, 1993). A type of diseases known as albinism is brought on by a decrease in the polymeric pigment melanin. Melanocytes, which originate from many ectodermal lineages, are typically extracutaneous (found in the eye and cochlea) or cutaneous (found in the skin and hair). In addition to albinism, oculocutaneous albinism (OCA) and ocular albinism (OA) are suitable to refer to specific disorders as well as to describe phenotypic characteristics. Albinism is first described by Sir Arcibald Garrod as an inherited metabolic defect.(Wildsoet, Oswald, & Clark, 2000)

It is now thought that OCA is a hereditary illness that is diverse and results from mutations in multiple genes. Numerous loci have been implicated in the genetic heterogeneity revealed by population research (Käsmann-Kellner B, 1998). Eight distinct genes can get mutated to cause one of the eight types of OCA (types 1-8). For instance, an alteration in the TYR gene causes OCA1, which is physically located on chromosome 11q14.3 (Shah, 2014), OCA2, which is physically positioned on the chromosome at positions 15q11.2–q12 and has 23 coding exons with 345 kb nucleotide sequences, is produced by a change in the P gene. This gene produces a 110 kDa protein with 12 transmembrane helices and 838 amino acids encoded. (Preising, 2007), The change in the TYRP1 gene creates OCA3, which is found on chromosome 9p23 and has a nucleotide sequence that is 17 kb long. It codes tyrosinase-related protein-1 (Tyrp1), which is 536 amino acids long, It has eight coding exons and is 61

kDa in size (Rooryck, 2008), OCA4 is induced by a mutation in the SLC45A2 gene (Memebrane Associated Transported Protein, MATP), located on chromosome 5q13, encompassing a nucleotide length of 40 kb and comprised of seven coding exons. It directs the synthesis of 530 amino acids via a membrane-cound transporter protein, SLC45A2, about 58 kDa in size, comprising 12 transmembrane helices (Inagaki, 2006), Oculocutaneous albinism type 5 (OCA5) is attributed to a mutation in an unidentified gene situated on chromosome 4 (4q24, including 14 genes) (Paige, 2014), whereas oculocutaneous albinism type 6 (OCA6) arises from an SLC45A2 gene mutation. (MATP), which is located on chromosome 15q21.1. (Wei, 2013), OCA7 is attributed to a mutation in the C10orf11 gene (LRMDA - leucine-rich melanocyte differentiation-associated gene), which is situated on chromosome 10 at the 10q22.2–q22.3 position. C20orf11 is a 226 amino acid protein that has three leucine rich repeats (LRR) domains and one LRC C-Terminal (Grønskov K. D., 2013) and OCA8 caused by a mutation in the dopachrome tautomerase gene, or DCT gene. Gene is positioned on 13q31–q32 of the chromosome. With eight coding exons, The TYRP2 Protein, which is 80% comparable to TYRP1, is controlled by the dopachrome tautomerase gene. (Sendoel, 2010) (Volk, 2021). The most frequent kinds of the disease are types 1 and 2, but types 3 and 4 are less frequent.

There are two types of oculocutaneous albinism genes: syndromic (HPS3, AP3B1, HPS1, HPS4, HPS5, HPS6, DTNBP1, BLOC1S3, PLDN, LYST, MYO5A, RAB27A, and MLPH) and nonsyndromic (SLC45A2, TYRP1, OCA2, and TYR) (Wei Li, 2006) (Ai-Hua Wei, 2013). Researchers define the genetic causes of OCA disorder brought on by mutations linked to the disease in this study.(Balu Kamaraj, 2014)

The types with fewer characteristics, the most chronic, OCA1A, has no formation of melanin at all over life, whereas OCA1B, OCA2, OCA3, and OCA4 have some pigment accumulation over time. The main causes of oculocutaneous albinism include mutations in OCA2, TYRP1, SLC45A2, and TYR. Two novel genes that produce OCA6 and OCA7, respectively, have recently been discovered: SLC24A5 and C10orf11. (Balu Kamaraj, 2014)

The majority of mutations linked to OCA that have been reported have been single base changes that cause nonsense or frameshift mutations, problems in RNA splicing, or alterations of amino acids. Chromosome rearrangements and larger deletions are significant methods for altering genes linked to OCA. A few of these OCA-causing genes are also implicated in the natural variance in skin and hair colour (Sturm, 2009). Variations in skin

and hair colour have also been linked to variations in the genes linked to OCA1, OCA2, OCA4, and OCA6. In Pakistan, marriage within families and high consanguinity are frequent causes of elevated rates of recessive illnesses, including OCA. In Pakistan, 62.7% of marriages are consanguineous, with 80% of these marriages being between first cousins.

Albinism has been well researched and affects individuals from many ethnic origins. Between one in 17,000 and 20,000 persons suffer from one of the forms of albinism. This indicates that the OCA gene appears in about 1 in seventy individuals Multiple founder mutations in different genes and the difficulty of clinically distinguishing between multiple albinism subtypes within the broad range of normal pigmentation contribute to the significant variations in prevalence of the various kinds of albinism across the world. The most prevalent type in the world is OCA2. (Grønskov K. E.-N., 2007).

In most groups, OCA1 is present at a frequency of around 1 in 40,000, however it is extremely rare in African-Americans. Conversely, OCA2 represents the predominant form of albinism among African Black individuals with OCA. In the United States, OCA2 prevalence is predicted to be 1:36,000 overall, but among African Americans, it is more like 1:10,000. In several areas of southern africa, 1 in 3,900 people are affected. OCA3, or rufous oculocutaneous albinism, is extremely uncommon in populations of Caucasians and Asians, but has been recorded to impact 1:8,500 people in Africa. Recently, it was discovered that OCA4, a fourth gene, is the cause of albinism. This gene mutation is thought to account for the condition in 20% of Japanese children with albinism but merely 5% to 8% of German patients (Grønskov K. E.-N., 2007).

X-linked hemophilia B is characterized by recurring episodes of soft tissue bleeding. The rare autosomal recessive disorder Hermansky-Pudlak syndrome is characterized by oculocutaneous albinism and a platelet storage pool abnormality that increases the risk of bleeding. This study describes a Caucasian guy with oculocutaneous albinism and hemophilia B who has a new mutation in HPS6. (Kevin J. O'Brien, 2017)

A rare autosomal recessive condition known as Hermansky-Pudlak syndrome (HPS) causes platelet abnormality (platelet storage pool defect), bleeding issues brought on by the storage of an aberrant fat-protein compound (lysosomal accumulation of ceroid lipofuscin), and oculocutaneous albinism (decreased pigmentation). Three medications for conditions linked to albinism have been discovered thus far. OCA1 and OA1 can be treated with L-DOPA

(Vanessa M Lopez, 2008)and Adeno associated viral vectors (AAV) (Gargiulo A., 2009). Nitisinone, a third authorized therapeutic medication, was first developed to treat individuals with hereditary tyrosinemia type. (Onojafe I. F., 2011)

Non-syndromic OCA and HPS are both autosomal recessive ailments, although having unique clinical features but some in common. Non-syndromic OCA and HPS are represented by reduced skin and hair pigmentation, nystagmus, iris transillumination, decreased visual acuity, increased optic nerve decussation, and foveal hypoplasia. (William A. Gahl, 1998)

The treatment has an advantageous effect because it enhances the concentration of the reagent utilized by the enzyme tyrosinase (TYR) by causing an unnatural build-up of tyrosine in the blood. With some tyrosinase activity still present, this results in enhanced pigmentation and greater oxidation in the skin and eyes of OCA1B rodent's models.Further, the Fukai group (Osaka, Japan) recommended the use of aminoglycosides as a suitable therapeutic intervention for a number of mutations often found in albinism. These aminoglycosides are known to promote the read-through response over certain nonsense mutations. (Fukai K., 2012)

Table 1Classification of albinism

Phenotype	Gene	location	Inheritance
OCA type 1A	TYR	11q14.3	AR
OCA type 1B	TYR	11q14.3	AR
OCA type 2	OCA2	15q12-q13.1	AR
OCA type 3	TYRP1	9q23	AR
OCA type 4	SLC45A2 (MATP)	5p13.2	AR
OCA type 5	OCA5	4q24	AR
OCA type 6	SLC24A5	15q21.1	AR

OCA type 7	LRMDA	10q22.2-q22.3	AR
OCA type 8	DCT	13q32.1	AR
OA type 1, Nettleship-falls	GPR143	Xp22.2	XL

CHAPTER NO: 02

LITERATURE REVIEW

Literature Review

OCA is an autosomal recessive genetic condition characterised by hypo-pigmentation of the skin, hair, and eyes due to either reduced or absent melanin synthesis. In OCA, the afflicted child receives a copy of the defective gene from each parent. The heterogeneity of OCA is made clear by the possibility that physically normal carriers of the disease can pass the disease on to the next generation due to such as just one defective gene copies or compound heterozygous changes in two separate OCA gene alleles, which have also been found in various communities everywhere (Oetting, 1998).

2.1 Clinical Features of Different OCA Types

OCA1 comes in two subtypes: OCA1A and OCA1B. While mutations that result in the retention of some enzyme activity generate OCA1B, where some melanin pigments accumulate over time, OCA1 is caused by a mutation that results in a total absence of tyrosinase function (Karaman, 2008). Tyrosinase type I, a 60 kDa glycoprotein, is encoded by the human tyrosinase gene (TYR, 11q14–q21, MIM 606933), which spans over 65 kb of genomic DNA and has 5 exons. (Kunal Ra, 2007).

The essential first and second reactions, first is the hydroxylation of tyrosine to L-DOPA and second is the oxidation of L-DOPA to DOPA-quinone are among the several processes in melanin production that tyrosinase catalyzes. Depending on residual activity, TYR mutations might result in either partial or total OCA (Dimitre R. Simeonov, 2013). A pseudogene called Tyrosinase-Like Gene (TYRL, 11p11.2; MIM#191270) is found on chromosome 11. This gene and the 3′-region of the TYR gene, which includes exons 4 and 5, have a 98.55% sequence similarity (Chaki, 2005). The TYR gene's initial pathogenic mutation was documented by Tomita et al. in 1989 (Yasushi Tomita, 1989).

The OCA1 variant with white skin, lashes, eyebrows, and hair is known as OCA1A. Over the course of a lifetime, colour does not change. Without regard to race or ethnicity, people with OCA1A have visual acuity of 1/10 or below throughout their lifetimes (Tripathi, 1993).
OCA1B, formerly known as yellow albinism, is another variety of OCA. The clinical features of this kind are comparable to those of OCA1A, except the skin and hair have sparse melanin pigment development. The iris's natural blue colour may change to a green or brown hue as a

result of increased pigmentation with ageing. The OCA1B type has a visual acuity of 2/10, while a different variety of OCA1 is temperature-sensitive OCA, in which non-pigmented body hair is initially present but may eventually develop pigmented hands and feet due to temperature differences between exposed and covered body parts (Lee, 2021).

Two OCA1 patients were found to have two novel harmful p.C89S and p.H180R mutations. Additionally, 17.5% and 35% of the patients, respectively, had the prevalent non-pathogenic SNPs R402Q and S192Y. The study's findings have broadened the range of OCA1 patients' genotypes. (Vadieh Ghodsinejad Kalahroudi, 2014)

OCA2, which is caused by mutations in the chromosome 15q11.2-12 P gene (Richard A. King, 2003), OCA2-affected people may have some melanin pigmentation, which can contribute to a wide range of skin, hair, and eye colours; nevertheless, the colour often stays lighter than in unaffected family members' cases (Manga, 2001). Typically, hair colour isn't entirely white. Those with OCA2 may have moles or pigmented nevi, as well as benign lesions or dark patches after prolonged sun exposure (Oetting W. S., 2005).

The mouse pink-eyed dilution gene (p), which codes for an integral membrane protein with 12 putative transmembrane domains, has been shown to be similar to human OCA2 (Eugene M. Rinchik, 1993). Although a number of possible roles have been suggested, such as a membrane transporter of a substrate, the P gene product's exact function is still unknown (Susana Rosemblat, 1998).

Table 2Mutations of the P gene in six Japanese patients with OCA2 (a) The mutation has already been reported, (b) These mutations are novel and (c) Second mutation was not identified (Tamio Suzuki Y. M., 2003)

Patient	Mutations detected	Clinical phenotype	Consanguinity
5	A481T (GCC>ACC)/IVS15+1 G>A	Mild OCA2	–
7	P198L (CCG>CTG)	Typical OCA2	–
13	P211L (CCG>CTG)/IVS24-1 G>C	Typical OCA2	–
21	R10W (CGG>TGG)/A481T(GCC>ACC)	Mild OCA2	–
33	M394I (ATG>ATA)	Severe OCA2	–
35	M394I (ATG>ATA)/A481T(GCC>ACC)	Mild OCA2	–

The six out of eight who retained their red hair after birth did so due to mutations in the MC1R gene, which caused their hair to be red instead of yellow or blond. This is the first evidence of a gene altering the human OCA phenotype. (Richard A. King, 2003)

Despite significant clinical overlap between OCA1A and OCA2, the symptoms of OCA2 individuals are often milder than those of OCA1A, underscoring the need of genetic investigation in the diagnosis of albinism. In addition to being mutated in OCA2, the P gene has also been linked to hypopigmentation and albinism in Prader-Willi syndrome. OCA 2 in Japanese patients was the main topic of this review. Only 8% of people have OCA2. However, almost 12% of those with normal pigmentation had the pathogenic p.A481T variant, which had 70% melanogenesis activity. This suggests that sub-clinical OCA2 may be more common in Japanese people than is generally believed. (Tamio Suzuki, 2008)

Oculocutaneous albinism type 3 (OCA3) is linked to mutations in the human TYRP1 gene and brown pelage in the mouse Tyrp1 gene. A gene product confined to melanocytes, tyrosinase-related protein 1 (Tyrp1) is involved in the production of eumelanin. Oculocutaneous albinism type 3 (OCA3) is linked to mutations in the human TYRP1 gene and brown pelage in the mouse Tyrp1 gene. Tyrp1 has significant dihydroxyindole carboxylic acid oxidase (DHICA oxidase) activity in the murine system (Rangaprasad Sarangarajan, 2001). TYRP1 is made up of eight exons and occupies 17 kb on chromosome 9p23.

OCA3, also known as rufous albinism, is an uncommon variant of red albinism that was initially identified in African populations. OCA3-affected people have reddish-brown complexion, yellow or reddish hair, and either brown (excessive melanin) or hazel (less melanin) eyes (Zhang, 2011). Examining the impacted instances of OCA1 and OCA2, thedegree of visual acuity impairment is less severe. Nystagmus and photophobia are two more significant characteristics of albinism that may or may not be present. (Bibi, 2020)

Tyrp1 is involved in regulating the catalytic activity of the tyrosinase protein and preserving its stability. Tyrp1 also influences melanocyte cell death and proliferation and maintains the ultrastructure of melanosomes. (Rangaprasad Sarangarajan, 2001)

Two novel mutations were find out in two Chinese patients, c.780-791del/c.1067G>A (p.R356Q) and c.625G>TT (p.G209LfsX1)/c.643C>T (p.H215Y). The c.780-791del and

c.1067G>A is already reported and these two c.625G>TT (p.G209LfsX1)/c.643C>T (p.H215Y) are novel mutation. To figure out the impact of these two mutations, bioinformatic analysis and population screening were conducted, and the results showed that both mutations were harmful. We hypothesize that OCA3 may be common in the Chinese community based on the moderate phenotype that these two individuals share. (Kai-hui Zhang, 2011)

OCA3 is an uncommon type throughout the globe, particularly in East Asia. There have only been two recent reports of Chinese patients thus far. The Japanese girl was identified to have compound heterozygous mutations p.C30R and p.367fsX384. Using in vitro tests, researchers then demonstrated that the missense mutation, p.C30R, was functionally incapable of melanin production. (Makiko Yamada, 2011)

Boissy et al. discovered in 1996 that an African American male with brown oculocutaneous albinism (BOCA) had homozygosity for a 1-bp deletion in the TYRP1 gene (c.1103delA, p.K368fs), which caused premature termination at codon 384. (R E Boissy, 1996)

Manga et al. then examined the TYRP1 of 19 unrelated black people from southern Africa who had rufous OCA (ROCA). They found that 17 of the 19 patients had a nonsense mutation (c.497C > G, p.S166X) and compound heterozygosity for c.1103delA, p.K368fs. Thus, OCA3 has been identified in individuals with albinism of African descent. (P. Manga, 1997)

The fourth pathogenic OCA gene was found to be a gene called "MATP" (membrane-associated transporterprotein) or "AIM-1" (antigeninmelanoma-1) that is found in chromosomal segment 5p. The 530 amino acid polypeptide that the human MATP gene produces has 12 putative transmembrane domains, is expressed in a significant proportion of melanoma cell lines, and shares structural similarities with plant sucrose-proton symporters. (Mamoru Harada, 2001)

The specific form of OCA known as "OCA4" was discovered in a single Turkish patient who had a homozygous G-to-A transition in the splice-acceptor sequence of exon 2 of the MATP gene (J.M. Newton, 2001).

The clinical characteristics of OCA4 and OCA2 are similar. The tissues in OCA4 patients exhibit a spectrum of pigmentation from brown to yellowish brown (Yousaf, 2020). In these situations, the severity of the visual acuity can range from 20/30 to 20/400, which is

correlated with melanin synthesis and manifests in tissue (Konno, 2009). In OCA4 instances, the visual acuity often falls between 20/100 and 20/200 (Sengupta, 2007).

One of the most frequent causes of tyrosinase-positive OCA in Japan is mutations in the MATP gene, as evidenced by the 24% (18/75) frequency of OCA4 in the Japanese albino population. 16 individuals were still categorized as having an unidentified form of OCA even after all PCR results derived from their DNA were directly sequenced to look for any mutations in the MATP gene. One of the most frequent causes of tyrosinase-positive OCA in Japan is mutations in the MATP gene, as evidenced by the 24% (18/75) frequency of OCA4 in the Japanese albino population. 16 individuals were still categorized as having an unidentified form of OCA even after all PCR results derived from their DNA were directly sequenced to look for any mutations in the MATP gene. (Katsuhiko Inagaki1, 2004)

OCA4 is typically identified in the first year of life due to nystagmus and strabismus in the eyes, as well as hypopigmentation of the skin and hair. After the early years, vision is probably stable. In OCA4, cutaneous pigmentation ranges from not much to almost normal. The hair of newborns with OCA4 typically has some pigment, ranging from pale yellow to silvery white. Hair color does not change much from childhood to maturity, however it may darken with time. (Hayashi M, 1993)

Biallelic pathogenic mutations in SLC45A2 are used in molecular genetic testing to diagnose OCA4 since its phenotype is similar to those of the other hereditary types of albinism (ocular and oculocutaneous). For this condition, the recommended molecular genetic testing approach is a multigene panel or complete genomic testing. As cumulative UV exposure is a significant risk factor for skin malignancies, people with OCA4 should avoid the sun from a young age. (Hayashi M, 1993)

To date, 18 pathological mutations have been identified. Eight Japanese individuals with a range of clinical symptoms were identified to have six new mutations: p.Y49C (c.146A > G), p.G89R (c.265G > A), p.C229Y (c.686G > A), p.T437A (c.1309A > G), p.T440A (c.1318A > G), and p.G473D (c.1418G > A). Like the other forms of OCA, OCA4 had a wide range of characteristics that most likely varied according to the SLC45A2 gene's mutation locations. (Katsuhiko Inagaki, 2006)

Family members affected with OCA5 have golden hair, white complexion, and ocular abnormalities similar to those of the OCA1 type. One member of the family that was impacted had a visual acuity of only 6/60 (Kausar, 2013).

Mutations at the 4q24 locus on chromosome 4 are associated with OCA5. While the exact gene linked to OCA5 has yet to be pinpointed, it is understood that individuals affected by it usually show symptoms akin to those found in other types of albinism. Especially OCA1, characterized by serious eyesight issues and light sensitivity. (Osama Al Deyabat, 2024)

Because OCA5 is rare, its prevalence is not well established; up to now, it has only been documented in a single family. In contrast, the prevalence rates of other forms of OCA vary according on the population. OCA1, for instance, is estimated to have a prevalence of about 1 in 40,000 people, whereas OCA2 is more frequent in specific populations, especially among African Americans. (Jennifer G R Kromberg, 2024)

In order to lower the risk of skin cancer, the main goals of managing OCA5 are to treat vision impairments and shield the skin from the sun. Concerned persons must have regular eye exams and UV protection as essential parts of their treatment. (Osama Al Deyabat, 2024)

Because there is little information available on Oculocutaneous Albinism Type 5 and no known causal genes, the disorder is still poorly understood. In order to better diagnose and treat people impacted, further study is necessary to clarify its genetic foundation and clinical characteristics. (Osama Al Deyabat, 2024)

Currently, there are no specific treatments or therapies that can cure Oculocutaneous Albinism Type 5 (OCA5). Management of the condition primarily focuses on symptomatic treatment and supportive care to enhance the quality of life for affected individuals. An ophthalmologist's routine eye exams are crucial. Refractive faults, which are frequent in people with albinism, can be corrected using prescription glasses or contact lenses. Magnifiers and telescopes are examples of low vision devices that can enhance visual skills and daily functioning. (Andrus, 2024)

Although they don't increase visual acuity, dark glasses or photochromic lenses are advised to reduce light sensitivity-related discomfort. Because of the higher risk of sunburn and skin cancer, people with OCA5 should be advised to protect their skin from the sun. (Andrus, 2024) (Osama Al Deyabat, 2024)

Oculocutaneous albinism (OCA) 6 is a non-syndromic form of OCA characterized by varied cutaneous hypopigmentation and unique ocular symptoms. SLC24A5, which codes for NCKX5, a K+-dependent Na+/Ca2+ exchanger 5, is the gene responsible for OCA6. Although its role in melanosome development is yet unknown, NCKX5 plays a role. We documented a Japanese patient with OCA6 in this study. Compound heterozygous variations were found in SLC24A5, c.590 + 1dupG, and c.598G>A (p.G200R) by genetic analysis. (Toru Saito, 2021)

Hypopigmentation with white skin, brownish irises, and a spectrum of hair colours from golden to dark brown define OCA6. OCA6's clinical characteristics are poorly understood as there aren't many instances studied globally that can't be distinguished by certain characteristics. One afflicted person's visual acuity was assessed at 20/100, and fundoscopic examination indicated hypo-pigmentation and an underdeveloped macula (Grønskov K. D., 2013).

The hallmark symptoms of OCA6 are caused by this mutation, which include white skin, transparent iridides, photophobia (sensitivity to light), nystagmus (involuntary eye movement), light hair at birth that darkens with age, and foveal hypoplasia (underdevelopment of the fovea) that lowers visual acuity. (Qurban Ali Shah, 2022)

One of the less common types of oculocutaneous albinism is OCA6. Oculocutaneous albinism is thought to affect 1 in 17,000 people worldwide, while actual frequency varies. Because OCA6 is rare, there is a lack of specific data on its prevalence. (Mervyn G Thomas, 2023)

According to a recent study, nystagmus, photophobia, strabismus, and blue irises were among the clinical characteristics of OCA6 instances that were not syndromic. The impacted individuals' eyesight was impaired, making it impossible for them to see without glasses (Konno, 2009).

Oculocutaneous albinism type seven (OCA7) is one of nine known non-syndromic types of albinism (OCA1-8 and OA1), and is one of the rarest forms of non-syndromic albinism.

Through homozygosity mapping in a consanguineous Faroese family with albinism2, Grønskov and colleagues were the first to find the LRMDA gene, which was then known as the c10orf11 gene, in 2013 (Karen Grønskov C. M., 2013).

The LRMDA gene, which codes for a 198 amino acid protein with three leucine-rich repeats (LRRs) and one LRR C-terminal domain, is found on chromosome 10 in the 10q22.2-q.22.3 area. Members of the family of proteins containing LRRs have a range of roles, such as RNA processing, neural development, and cell adhesion and signaling. (J. Bella, 2008)

Skin hypo-pigmentation and hair colour variation ranging from white-golden blond to dark brown are features of OCA7 clinical presentations in children whose parents or siblings are carriers and normal. The afflicted patients had impaired visual acuity, ranging from 6/18 to 3/60, and other ocular issues related to nystagmus and iris trans-illumination were noted (Pennamen, 2021).

OCA7 individuals had a severe ocular phenotype with VA at the lower end of the albinism continuum, significant foveal hypoplasia, and chiasmal misrouting, despite minimally altered pigmentation levels. Patients with OCA7 had an eye-specific phenotype that was comparable to that of X-linked ocular albinism. As a result, scientists suggest renaming the condition ocular albinism type 2. Determining the function of LRMDA in OCA7 might help us pinpoint the causes of the co-occurrence of misrouting and foveal hypoplasia. Researchers discovered one new mutation in the Dutch patient in this study: c.565G > A; p.(Gly189Ser). (C. C. Kruijt, 2024)

The amount of pigmented melanocytes, dopachrome tautomerase (DCT), melanoblasts, and pigmentation all decreased when the LRMDA zebrafish homolog was knocked down. An essential enzyme in the route leading to the creation of melanin is DCT (Karen Grønskov C. M., 2013). Dopachrome cannot be changed into dihydroxyindole carboxylic acid (DHICA) without DCT. Dopachrome spontaneously transforms into dihydroxyindole (DHI) when a portion of the eumelanin production pathway is inhibited. It is still possible to synthesize eumelanin when tyrosinase is present (Mirosława Cichorek, 2013). This might be the cause of the fact that, despite LRMDA's significance for melanocyte growth and differentiation, mutations in neither zebrafish nor humans completely eliminate pigmentation. (Karen Grønskov C. M., 2013)

The main cause of oculocutaneous albinism type 7 (OCA7) is mutations in the C10orf11 gene, which codes for a protein involved in the manufacture of melanin. These are the main OCA7 mutations that have been found. The frameshift mutation c.66dupC causes an early stop codon, which truncates the protein. Patients from a consanguineous Kurdish family have been found to have it. Two mutations, c.565G > A and c.566G > A, have been found in a

Dutch patient who is a compound heterozygote for them. They cause amino acid changes (p.Gly189Ser and p.Gly189Asp, respectively). (C. C. Kruijt, 2024)

Researchers presented the first proof that two unrelated individuals had OCA due to DCT mutations. Notably, both patients experienced a modest reduction of visual acuity along with minor hypopigmentation of the skin, hair, and eyes. Despite the clinical heterogeneity of albinism, DCT patients appear to be on the milder end of the spectrum. Clinicians may fail to notice these subtle symptoms, which might result in an underdiagnosis of albinism. Gene panels for diagnosing albinism should now contain the DCT gene. Researchers suggested that OCA8 is the name of the equivalent version of OCA. (Perrine Pennamen, 2020)

Two patients were found to have variations in the Dopachrome tautomerase (DCT) gene. One was compound heterozygous for c.118T>A p. (Cys40Ser) and a 14 bp deletion in exon 9. The second had c.183C>G p. (Cys61Trp) homozygosity. Both individuals showed traditional ocular characteristics and modest hypopigmentation of the skin and hair. (Perrine Pennamen, 2020)

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Researchers found that all three afflicted family members had a homozygous form of the missense mutation c.176G > T (p.Gly59Val) in DCT. Researchers demonstrate that this mutation causes in vitro targeting and inadequate protein maturation, which can lead to a partial or complete loss of function. Following screening of a cohort of patients with INS (n = 25) and OCA (n = 85), two heterozygous truncating variants were found in an independent OCA patient: c.1407G > A (p.Trp469*) and c.876C > A (p.Tyr292*). When combined, our findings imply that DCT mutations can result in a range of phenotypes, from oculocutaneous albinism to isolated infantile nystagmus. (Alexander E. Volk, 2021)

The SLC45A2 gene, which is essential for melanocytes to produce melanin, is mutated in OCA8. A protein involved in the transportation of chemicals required for melanin production is encoded by the SLC45A2 gene. The distinctive characteristics of OCA8 are caused by mutations in this gene that interfere with normal pigment synthesis. (Mervyn G Thomas, 2023)

OCA8 is a milder form of albinism characterised by moderate characteristics such as iris illumination, nystagmus, moderate foveal hypoplasia, and hypopigmentation of the skin and hair (Volk, 2021).

OCA8's clinical features are described by: Compared to their contemporaries, people with mild hypopigmentation usually have lighter skin and hair, but the pigmentation is not as severely compromised as in other forms of oculocutaneous albinism and Visual Impairments: Foveal hypoplasia (underdevelopment of the fovea), decreased visual acuity, and nystagmus (involuntary eye movement) are common ocular signs. Additionally seen are retinal hypopigmentation and iris transillumination. The frequency of symptoms Although each person's symptoms might vary greatly, those who are affected often have a slight decline in visual acuity and may be more susceptible to photophobia (light sensitivity) and other vision-related problems. (Mervyn G Thomas, 2023)

Nearly every incidence of OCA in southern Africa has been identified, along with two less common types, rufous OCA (ROCA) and brown OCA (BOCA). This group does not seem to have tyrosinase-negative OCA (OCA1, or ty-neg OCA), which is brought on by mutations in the tyrosinase gene (TYR) (Ramsay M, 1992). A 2.7-kb deletion is the most frequent mutation (D. Durham-Pierre, 1994), which, in southern Africa, is responsible for 78% of OCA2 mutations. (Stevens G, 1995)

Skin and hair color are the primary physical characteristics that differentiate Brown Oculocutaneous Albinism (BOCA) from Rufous Oculocutaneous Albinism (ROCA) (Kromberg, 1990). The Nigerian people were the first to define BOCA. It has been discovered to exclusively be present in Black, or Negroid, populations in America and Africa (Richard A. King R. A., 1985). The majority of ROCA cases have been documented among Black communities in Papua New Guinea and Africa. (Kromberg, 1990)

The frequency of ROCA in southern Africa is around 1 in 8,500 people. The disorder causes blue or brown irides, ginger-red hair, and reddish-bronze complexion. Approximately 76% of ROCA people have nystagmus, one of the relatively minor visual abnormalities linked to albinism. (Kromberg, 1990)

Table 2 lists the clinical criteria used to categorize the various forms of albinism. The characteristics were chosen based on the descriptions provided by (RA King, 1994) and

(Kromberg, 1990). Individuals who did not precisely fit the requirements were included in the mutation-detection investigations but excluded from the linkage research.

Table 3Criteria for the definition of BOCA and ROCA

Traits	BOCA	ROCA
Color of Skin	Light brown or tan	Bronze, mahogany, or brick-red
Color of Hair	Pale brown	Reddish-ginger or ginger
Nystagmus	Absent or mild	Mild or absent

2.2 Prevalence of different OCA types:

The estimated frequency of OCA prevalence is 1 in 17,000–20,000. The OCA2 mutant allele is present in around 1 in 70 people and is considered the most common kind of albinism globally, even though the OCA1 phenotype has a greater frequency of mutation detection. With a rate of 1 in 1000 instances, OCA2 alleles are more common in the sub-Saharan African population (Lee S. T.-K., 1994). The prevalence of the various forms of albinism varies among populations. Type 1 OCA is the most prevalent kind (70% of cases) in America, China (sporadic albinism), the subcontinent (mainly familial albinism), and the Caucasian population. Its incidence is about 1 in 40,000 globally (Liu, 2014).

With a prevalence incidence of 1 in 39,000 globally, OCA2 is the most prevalent kind. With an approximate prevalence of 1 in 3900, this kind of albinism is most common in sub-Saharan Africa, followed by Americans and African-Americans (Manga, 2001). In addition, families with numerous genetic abnormalities in Pakistan and India also experience these two forms of OCA (Sajid Z, 2021).

OCA types 3 and 4 have a frequency of 1 in 8500 and 1 in 100,000, respectively. There have been reports of OCA3 in Indo-Pakistani, Japanese, and German populations. OCA4 is prevalent in the Japanese population, accounting for 24% of all cases. Other nations in Asia and Europe follow suit There is just one known consanguineous Pakistani family with OCA type 5 (Kausar, 2013).

Although there have been reports of OCA6 and OCA7 instances in China, eastern India, and Atlantic Islands (Veniani, 2016) (Yousaf, 2020), the precise incidence of these conditions has not been determined.

OCA often comes on by mutations in the TYR gene, which codes for the tyrosinase enzyme. A 53-year-old male proband with typical OCA symptoms, such as depigmentation of the skin, hair, iris, and fundus, as well as photophobia, reduced vision, nystagmus, macular fovea hypoplasia, and cataracts, was found to have TYR gene variations in a Chinese family study. The significance of thorough clinical and genetic investigations in the diagnosis and treatment of OCA, as well as in offering genetic counseling to impacted families, is highlighted by this example. (Wang, 2024)

The particular genetic alterations causing OCA determine its phenotypic expression. OCA1, OCA2, and OCA4 subtypes did not significantly differ in binocular visual acuity, according to a study that examined 127 patients with genetically verified OCA. Nonetheless, variations in refractive errors were noted among these types. This study underscores the need for customized clinical evaluations and the wide range of phenotypic heterogeneity linked to various OCA genotypes. (Seguy, et al., 2023)

OCA4, which is caused by mutations in the SLC45A2 gene, has been documented worldwide but is more common in Asian people. Two main phenotypes were identified from a study of thirty OCA4 patients: one with milder characteristics and one that resembled the traditional OCA1 appearance. At least one missense mutation in SLC45A2 was frequently linked to the milder phenotype, indicating a link between certain genetic variations and clinical severity. (Ester Moreno-Artero, 2022)

A study of 40 OCA families in Pakistan found that TYR and OCA2 gene variants were common. Individuals that were affected showed common symptoms of OCA, including nystagmus, photophobia, and skin and hair hypopigmentation, most notably had irises that were grayish-blue. As part of the mutational spectrum of OCA in this group, the study found a number of recurrent mutations, such as p.Cys35Arg, p.Arg278*, and p.Gly419Arg in TYR, and p.Asp486Tyr and c.1045-15T>G in OCA2. (Thomas J Jaworek, 2012)

Nonsyndromic albinism includes an ocular variant (OA1) and eight oculocutaneous variants (OCA1–8), whereas syndromic albinism include.Chediak-Higashi syndrome and Hermansky-

Pudlak syndrome (11 variations, HPS1–11).7. All forms aside except OA1, which is X-linked are autosomal recessive. (Seguy, et al., 2023)

Incorrect refraction, ocular symptoms of albinism comprise diminished visual acuity, photophobia, strabismus, nystagmus, iris transillumination, retinal hypopigmentation, foveal hypoplasia, and abnormalities of the optic nerve head. Molecular biology-based diagnostic methods are needed to determine the exact kind of OCA. (XiaoFei Chen, 2023)

A tyrosinase-negative (ty-neg) albino displays characteristics such as pink skin, white hair, a pronounced red reflex, severe nystagmus, photophobia, and a visual acuity defect that remains constant regardless of age or race. Hair bulbs that are incubated in tyrosine do not produce pigment. (CARL J . WITKOP, 1973)

Phenotypic characteristics of tyrosinase-positive (ty-pos) albinos differ based on age and parental pigmentation levels. The majority of Caucasian typos, as well as albinos across all age groups, and infant Negro and Amerindian albinos, exhibit phenotypic similarities to ty-neg albinos. Tiny the amounts of pigment gather in the skin, hair, and the eye as we old. Patients may have crucial nystagmus and photophobia, pigmented nevi, a loss of the obvious red reaction, flaxen to yellow hair, a very slight tanning capacity, and even brown irides. Tyrosine exposure releases pigment in hair bulbs. (CARL J . WITKOP, 1973)

CHAPTER-03

MATERIALS AND METHODS

3.1 Fieldwork:

3.1.1 Ethical Approval and Legal Statements;

This study was initiated after obtaining prior ethical approval from the ethical committee of research, Faculty of Sciences, IIUI. Before the study, every family included in this present study received education on the possible results., advantages, and risks if any regarding this research. Then a written and informed consent form was obtained from the affected individual or their legal guardian.

3.1.2 Families' Identification and Enrollment;

Families with oculocutaneous albinism are identified from different regions of Pakistan. An extensive interview was taken with the elders of the family for clinical history and history of the disease in their families. A family pedigree was drawn for each family.

3.1.3 Clinical Assessment:

Individuals undergo thorough clinical assessments by specialized professionals for albinism. This includes standardized diagnostic tools and clinical findings. Interviews with families gather comprehensive clinical history and pedigree information and cover developmental milestones, medical history, family oculocutaneous albinism disorder history, environmental exposures, and socio-demographic factors.

3.1.4 Pedigree Analysis;

Pedigrees were drawn for detailed analysis of inheritance patterns.Haplopainter32 software was used for drawing pedigree, where the males were represented by a square and the females by circular signs. Furthermore, the affected male's and female's signs were shaded all respectively, while normal individuals were shown as unfilled signs. Deceased individuals were represented by drawing a diagonal line across a square/circle to distinguish them from live individuals. Consanguinity was represented by a double line between couples.

3.1.5 Blood Sampling;

Blood from selected families was taken in 5ml EDTA (Ethylene Diamine Tetra Acetic Acid; an anticoagulant that chelates Ca2+ ions)) tubes using 10cc disposable sterile syringes. The afflicted people, their parents, and one of their conventional siblings all had blood samples obtained. Blood samples collected from the unaffected family members were taken as

controls. The collected samples were brought to the Human Molecular Genetics Lab at the Department of Biological Sciences, IIUI, Pakistan to store at 4°C for further analysis.

3.2 Lab Work

3.2.1 DNA Extraction:

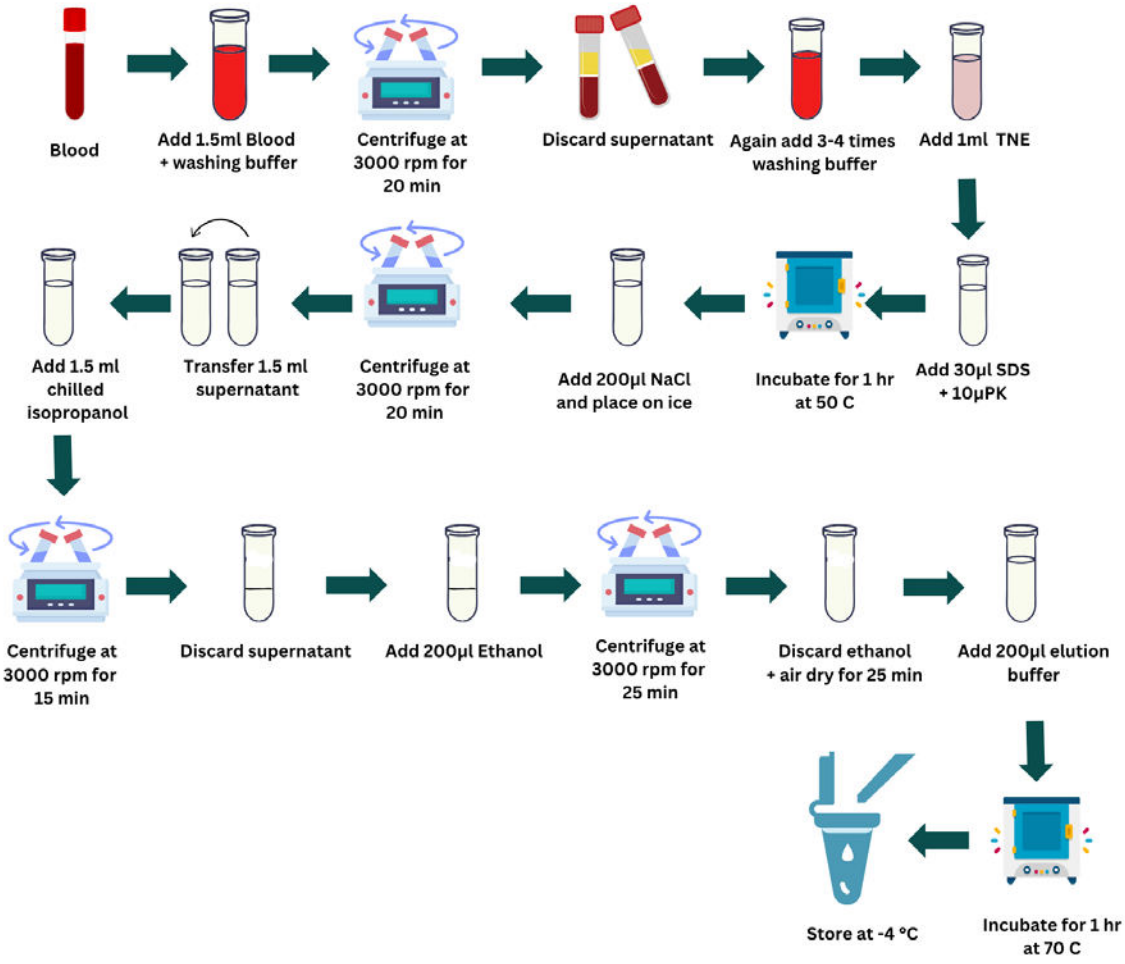

Figure 1In-organic DNA extraction method, (extracted from Sajida Hadayat, 607-FOS/MSBT/S23, MS Thesis)

In-organic DNA extraction protocol was followed to extract the DNA. (Waqar Arshad, 2018)

1. 1.5 ml blood from each sample was taken in separated 15ml falcon tubes.
2. To lyse the RBCs, blood samples were frozen.
3. Each sample was combined with 13 ml of washing buffer, TE buffer, and Tris-EDTA buffer (10Nm Tris HCL pH8, 2Mm EDTA) in falcon tubes.
4. These were centrifuged at 3000rpm for 20 minutes at 25oC.

5. The particle was broken by gentle tapping and vortexing, and the supernatant was carefully discarded of using the pipette.
6. Until the WBCs' pellet was devoid of hemoglobin (RBCs), the washing procedure was carried out three or four times. Up to the 2-milliliter point, the supernatant was thrown away.
7. The pellets were resuspended in 1ml of TNE buffer (1OmM Tris HCl pH8, 2mM EDTA, 400mM NaCI), 30µl of 10% SDS(a detergent that helps in the breakdown of cell membranes), and 10µl of Proteinase K(protein digesting enzyme) were added.
8. The samples were mixed gently for 30 seconds and incubated at 50°C in the incubator for 1 hour in the water bath. (This helps the SDS and Proteinase K to break down proteins and other contaminants).
9. Samples were taken out and 200µl of saturated NaCl (6M) was added to each tube.
10. Sample tubes were shaken vigorously and then placed in a freezer for chilling on ice for 20-25 minutes.
11. The samples were taken out from the freezer and left for 5 to 6 minutes for melting at room temperature. The sample tubes have been centrifuged for 30 minutes at 25 degree celcius at 3000 rev/min.
12. Carefully 1.5ml supernatant from each tube was taken out in a new, clean tube.This contains the DNA in the solution and the pellet containing protein was discarded.
13. Noe equal amount of chilled isopropanol was added to the tube, by gently inverting the tube DNA became visible in the form of white threads in the solution.
14. Samples were centrifuged at 3000 rpm for 15 minutes at 25 °C after 10 minutes. The DNA pellet remained intact while the supernatant was properly disposed of.
15. 200 µl ethanol was added to the pellet to wash any remaining impurities. Centrifuged and ethanol were discarded carefully.

16. Tubes were placed upside down on tissue paper for 20-25 minutes to air-dry the DNA.
17. 200 µl elution buffer was added to the dried pellet, to dissolve and stabilize DNA.
18. The tubes were left in an incubator at a temperature of 70°C to render any extra nucleases dormant.
19. For additional assessment, all of the dissolution tubes were placed in Eppendorf tubes with the appropriate labels and kept in the freezer at -4°C.

Table 4Chemicals used and their role in DNA extraction

S.N0	Chemical used	Role in extraction
1.	Tris-EDTA	EDTA; Chelating agent(binds with Mg-ions, nullifies the action of DNase) Tris; cell lysis, maintain stable ph.
2.	TNE buffer	Stabilize DNA
3.	SDS	Detergent, Breaks down cell membranes and denatures proteins.
4.	Proteinase K	Protein digesting enzyme
5.	Saturated NaCl	Precipitate proteins and help separate them from DNA
6.	Isopropanol	Precipitate DNA and chilled alcohol increase the yield of DNA, helping DNA to not dissolve in water.
7	Ethanol	Washes DNA pellet, removes salts

3.3 Quantification of Extracted DNA

Extracted DNA samples were quantified through 1% agarose gel electrophoresis.

3.3.1 Gel Electrophoresis:

3.3.1.1 Gel preparation

A conical flask containing 60ml of 1×TBE buffer was then filled with 0.8 g of agarose powder. The solution was properly mixed by stirring the flask thoroughly. The flask was heated on a micro-oven for 30 seconds to 1 minute until bubbles appeared. Bubbles formation shows that agarose is dissolved in the buffer solution. The solution was cooled for 1 to 2 minutes.2µl EtBr was added. Then in clean gel caster solution was poured. Combs were placed in the caster. After 25-30 minutes the gel was prepared in semi-solid form. Gel Comb were removed and the gel-containing wells were ready for the electrophoresis process.

3.3.1.2 Gel electrophoresis procedure

Using agarose gel electrophoresis, a process that uses ultraviolet-induced fluorescence of Ethidium Bromide dye with tested nucleic acid, qualitative analysis and the amount of extracted DNA samples were ascertained. This technique compares the fluorescence of test or experiment DNA with that of standard DNA, and the amounts of fluorescence and nucleic acid are always in proportion to one another.The next step was utilizing ethidium bromide to prepare a stained 0.8% Agarose gel slab. 5µl quantity of DNA sample was mixed with 10 µl of loading dye (which is Bromophenol Blue 2X) and 5 µl of distilled water, were applied in gel, and after 25 minutes with the help of 120 V were resolved. For documentation purposes gel slab was exposed to a UV cross illuminator (gel documentation apparatus). The amount of every sample was measured and documented in the DNA extraction forms.

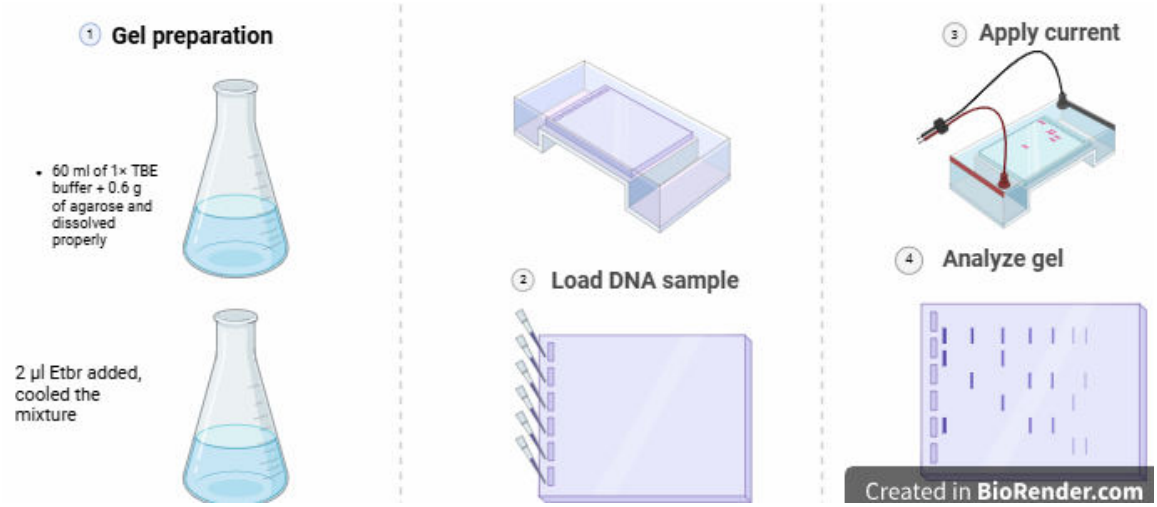

Figure 2In-organic DNA extraction (figure from BioRender.com)

3.4 Whole Exome DNA Library Preparation:

The Thruplex DNA-Seq (Rubicon Genomics) Library Preparation kit and the Agilent Technologies SureSelect V5 Exome Capture Kit were used for whole exome sequencing (WES). The Covaris ME220 Focused-Sonicator was then used to further shear the DNA, resulting in a sheared size of about 200 bp. After electrophoresis, the Agilent 2100 BioAnalyzer System was utilized to assess and determine the fragment length distribution of the DNA samples.

Library preparation:

1. Fix and dA-Tail the ends of the DNA.

2. Bind the P5-indexed adaptor.

3. Perform AMPure XP bead purification of the sample.

4. A major goal is to amplify the adaptor-ligated library.

5. To put it briefly, use AMPure XP beads to purify the amplified library.

6. Quality and quantity are done using the sample preparation protocol to get DNA libraries for sequencing in the Illumina paired-read platform. Staining of the sample is possible using either the 2100 Bioanalyzer instrument or an Agilent TapeStation instrument. The method of library preparation is done in such a manner that for each sample, an individual dual-index library is created.

Hybridization and Capture:

These prepared gDNA libraries are hybridized with a target-specific probe. The hybridization reaction is carried out using 500 – 1000 ng of the prepared DNA, in 12 µl volume. Utilize the maximum concentration of the prepared DNA within the range of this experiment.

1. DNA samples are hybridized to the probe.

2. Magnetic beads are prepared with streptavidin coating.

3. The hybridized DNA was then retrieved using streptavidin-coated beads.

Processing Post-Capture Samples for Multiplexed Sequencing:

The SureSelect-enriched DNA libraries undergo PCR amplification in this step.

1. The captured libraries are amplified.

2. Using AMPure XP beads purify the amplified captured libraries

3. Determine the sequencing library DNA yield and the quality of the library DNA.

4. Pool samples for multiplexed sequencing.

5. Sequencing samples are prepared.

6. Sequencing performs and assesses the data being examined.

3.6 WHOLE EXOME SEQUENCING (WES)

Exome sequencing will be conducted as well to create a representation of private, novel variants that are absent from any genetic database or RS.Coding regions were sequenced on the HiSeq2000 platform with 2 × 100 paired-end reads at a mean coverage depth of 30X at Otogenetics corporation, Norcross, GA, USA. For the final analysis, target-enriched libraries were sequenced for complete trios using the base facilities of CAMH, Toronto, with the exome enrichment Agilent Sure Select Human All ExonV4 (51 Mb) kit. For the three research sites, the next-generation sequencing platforms were Ion Proton using the Ion AmpliseqTM Exome kit and SOLiD 5500 using a technique.

3.8 Variant Annotation, Filtering, and Prioritizing

For each patient, the VCF files created were transferred to VarSeq® where each genetic variant was annotated. The SNVs were detected using a VarSeq-CNV® caller algorithm. To detect CNVs the algorithm works with depth of coverage inherent in each patient BAM file using data on BMs from a certain set of “reference” samples which were found not to contain CNVs in previous studies. CNVs were identified and called based on elevated ChiPSequenceReadDensity, suggesting high amounts of sequence duplication, and reduced ChiPSequenceReadDensity suggesting sequence deletion. Whole exome sequencing data was processed based on known bioinformatics pipelines to filter out or highlight the low frequency, de novo, or compound hetero/homozygous variants. For the Discovery and Restoration population sets, Golden Helix VarSeq Software was used to further process and

analyze the variant call data, which are saved as Sentieon® derived variant call files (VCF). Variants were processed to cover only one base pair position after assessing the variant quality control measurements, the frequency, and the pathogenic predictions of each variant low-quality variations in the Ingenuity Variant Analysis. (Note, all variants that were flagged by our filters for further evaluation if any of them was reported in ClinVar before, were filtered out after annotating all identified variants using this software and building custom steps and filter chains.) However, to test our filtration steps, as described below in the work variants In ClinVar they were taken into account only at some later stage, after undergoing the filtration procedure. The subsequent step involves filtration based on the mode of inheritance as follows: It's either autosomal recessive, autosomal dominant, or sex-linked. Next, we categorized the genotype as homozygous, heterozygous, or hemizygous, where the latter is appropriate in cases of XL inheritance. Other classes of inheritance that are not included in this work include mitochondrial or digenic. In addition, due to the limitation of WES, we could not determine variants in noncoding regions of the genome. Last but not least we searched for the consequences of the variant at the transcript level. While making this assessment, we made distinctions based on some of the worst effects which include loss-of-function [LOF], missense, silent, and intronic. According to ACMGG standards, we categorized variants as pathogenic/likely pathogenic. Variant assessments of the clinical course of the variant also contained clinical data with physical examination, additional laboratory studies/imaging, segregation analysis, genotype/phenotype correlation, previous publication, or de novo reassessment of the variant. Outcomes were deemed "Positive" for; that based on the ACMGG guidelines, a disease-causing variant has been called.

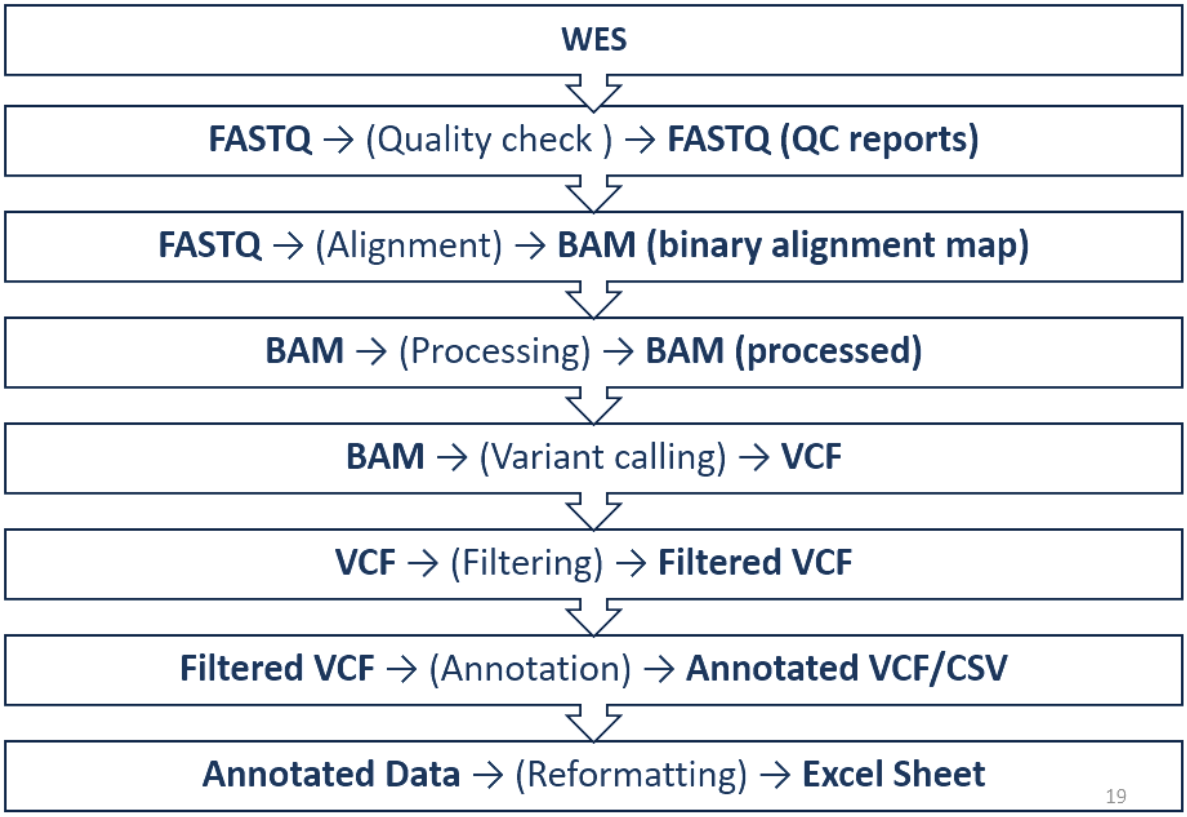

Figure 3Work flow for variant calling and annotation.

3.5 Excel Sheet Analysis:

Different filters are applied to short list our candidate variant: like,

Exclude variants with low read depth <10–20 reads.

Focus on rare variants with minor allele frequency (MAF in population databases like gnomAD.

Prioritize our variants on the basis of mutation type

Focus on genes associated with OCA

ClinVar: variants labeled as pathogenic or likely pathogenic

Validate candidate variants against literature

3.6 IN-SILICOPREDICTION OF EFFECT FOR MISSENSE MUTATIONS

Finally, these chosen variations were being used for in-silico computational pathogenicity prediction frameworks. We apply prediction tools like SIFT, PolyPhen2, mutation taster, ClustalW to determine if putative missense variations are likely to be damaging to the protein function and hence potentially disease-related.

An in silicotool Polymorphism Phenotyping v2 predicts if the missense variants are likely to affect the function of the protein based on the multiple sequence alignment supplemented by any available information about the named protein. Its score ranges from 0-1 indicating;

1. Scores between **0.0 and 0.15** are thought to be innocuous.
2. Scores between **0.15 and 1.0** might be harmful.
3. It is more certain that scores between **0.85 and 1.0** will be harmful

The visual indication is presented with a gradient from green, predicting benign, to red, predicting damaging variants.

3.6 3D-Modeling of Mutations

We can learn about the impact of mutations on proteins through in-silico study of mutant proteins. To assess the impact of mutation in this work, a normal protein structure and a mutant protein structure were created using Swiss modeling.

3.12 Conserved Regions;

Conserved region of the candidate genes are studied in different species. ClustalW multiple alignment sequence of OCA2, from two different families shows that all twovariants (p.R720C) and (p.T720M) are evolutionary conserved among different species (red mark represent the conserved amino acid which got mutated in our probands).

CHAPTER NO 04

RESULTS& DISCUSSIONS

4.1 Family- IIU-OCA-10 and IIU-OCA-08

The closely related IIU-OCA-10 family is located in Khyber Pakhtunkhwa province of Pakistan, in this family two affected females with different age groups, one is grand-daughter and other is the grandmother of her. At the time of initial examination, she (granddaughter) exhibited symptoms of night blindness, photophobia, and nystagmus, and passed away. (Figure 4.1.1).

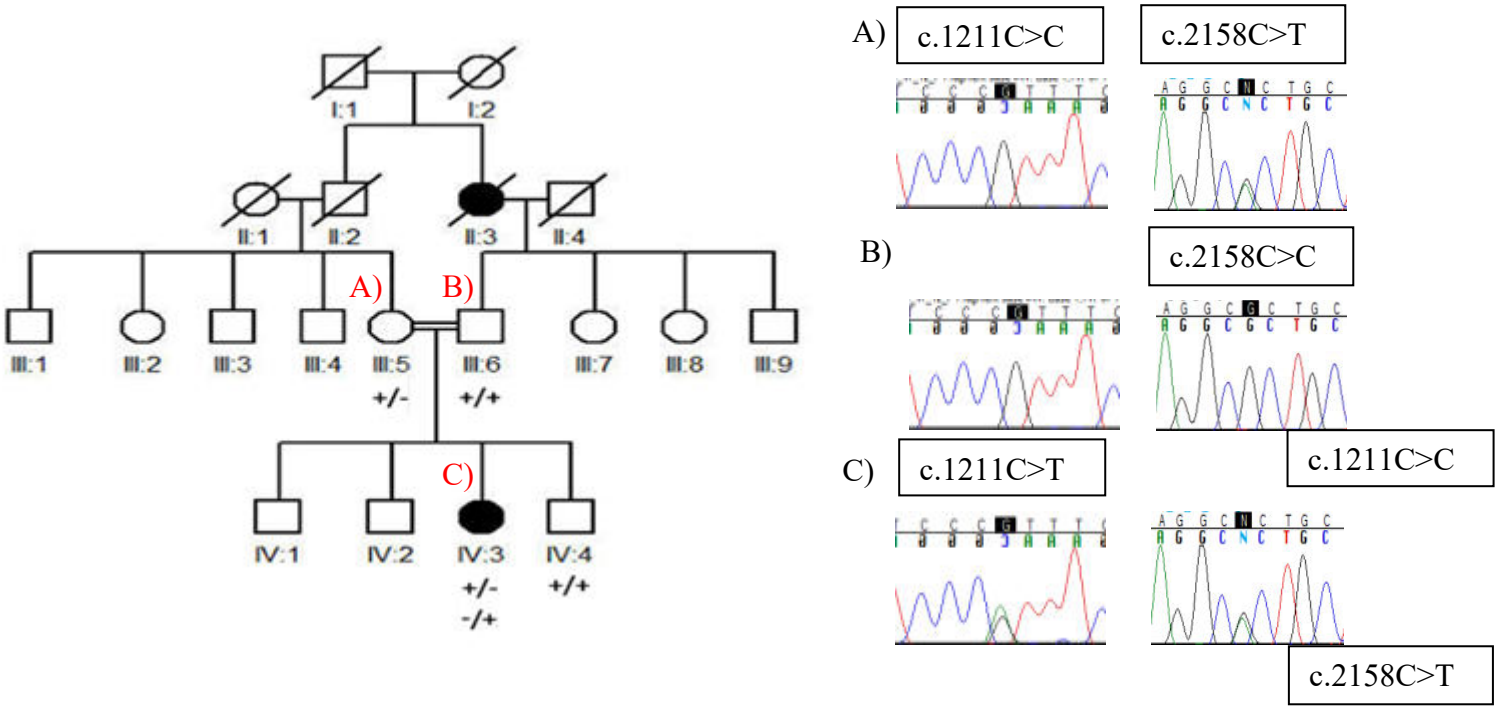

Figure 4Pedigree of family Family-10: showing two affected individuals (two females) parents of all affected person were normal.

The family- **IIU-OCA-08** a consanguineous family also belongs to Khyber Pakhtunkhwa province of Pakistan in this family two affected males and one female with different age groups, two are siblings, sister and brother and one is their uncle. At the time of initial examination, they had the signs of Nystagmus, Photophobia and night blindness. (Figure 4.4.2)

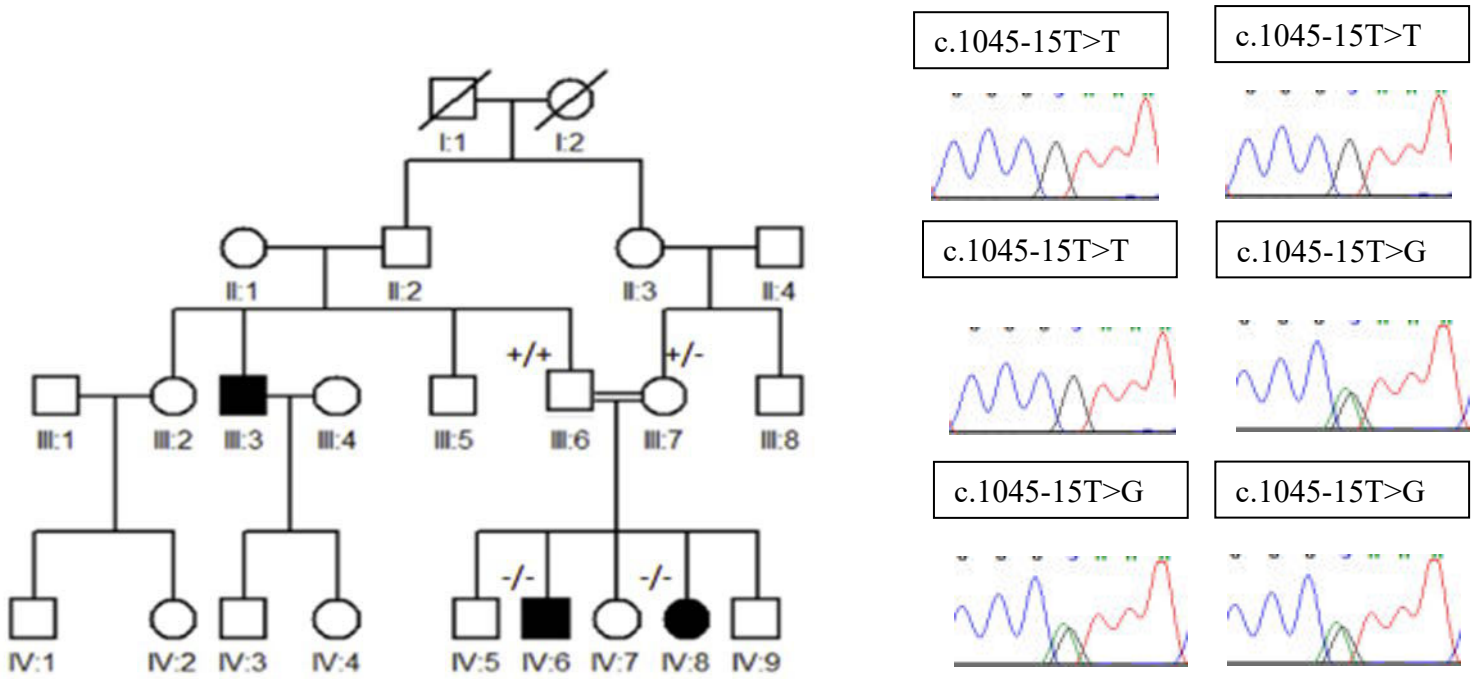

Figure 5Pedigree of family Family-IIU-OCA-08: showing three affected individuals (two males and one female) parents of all affected person were normal

4.1.1 Molecular Genetic Studies

4.1.1.1 Method

For two families, next-generation sequencing was done. both IIU-OCA-08 and IIU-OCA-10. Each family's impacted member was chosen, and the Illumina TruSight was used for analysis. A single panel for clinical exome sequencing (Ammann et al., 2016)

4.1.1.2 Genetic Finding

Exome sequencing identified two three reported *OCA2* variants in this study in family 08 and 10.

IIU-OCA-10 sequencing identified compound heterozygous variant chromosome no 15*(GRCh38): g.28116385C > T; c.2158C > T; p.Arg720Cys,* and *c.1211C > T; p.Thr404Met*. these two mutations are already reported in Pakistani population (Figure 4.3.4) and othervariant chr2 *(GRCh38)*: g. 238461079G > A; NM_024101.7: c.1776G > A; p.Ala592= ; this is not disease causing, a silent mutation.

In IIU-OCA-08 sequencing identified intronic variant which may alter splice acceptor site and its clinical significance is likely pathogenic, variant is c.1045-15T>G, which is present in 9th intron and 15 bp upstream to exon 10.

4.1.1.3 Clinical re-evolution

After genetic testing, all those impacted had a clinical revision. The cardinal clinical characteristics of OCA, including white hair, pale skin color, and nystagmus, were present in all afflicted people. They also reported experiencing photophobia and having reduced visual acuity. Clinical examination showed a hypopigmented albinotic fundus on direct ophthalmoscopy and foveal hypoplasia in all afflicted individuals. Complete clinical data for one family member who is impacted is summarized in table 4.3.5.

Table 5Summary of clinical re-evolution of family- IIU-OCA-10 and IIU-OCA-08.

Parameters	IIU-OCA-10	IIU-OCA-08
Age (Years)	10	08
Gender	Female	Female
Region, Province	KPK	KPK
Cast	Khan	Yousufzai
Hair Color	White/brown	White/brown
Skin Color	White	White
Skin Rashes	Absent	Absent
Skin Pigmentation	De-pigmented	De-pigmented
Eye Color	Brown	Blue
Visual Acuity	Decreased	Decreased
Nystagmus	Present	Present
Wave Form	Jerk	Jerk
Iris Color	De-pigmented	De-pigmented

Photophobia	Present	Present
Foveal Hypoplasia	Present	Unable to determine
Fundus	Albinotic	Albinotic

4.1.1.4 In-Silico analysis

In silico prediction methods including SIFT, PolyPhen-2, Mutationtaster, and PROVEAN were used to assess the pathogenicity of the variations that were found. When taken as a whole, these evaluations provide valuable insights on the pathogenicity of the variations. (Table 4.3.8).

Table 6In-silico prediction analysis to confirm the pathogenicity of the variants of IIU-OCA-10 and IIU-OCA-08.

Family	**IIU-OCA_1O**	**IIU-OCA_08**
Nucleotide Variant	c.1776G>A c.2158C>T c.1211C>T	c.1045-15T>G
Protein Variant	p.Ala592= p.Arg720Cys p.Thr404Met	-
Status	- Compound Heterozygous	Intronic Variant
Type of Mutation	Silent Mutation Mutation (Missense Mutation)	May alter splice site
Previously Reported	Yes	Yes
Polyphen-2	- 0.997 0.999	-
Mutation Taster	Deleterious Deleterious	-
SIFT	-0	-

ClinVar	No Affect Pathogenic pathogenic	-
Region	Splice site Variant CDS CDS	Intronic
Frameshift	No	No

4.1.2 Structural analysis

4.1.2.1 OCA2 gene

Human OCA2 does not have a crystal structure in the Protein Data Bank . The protein structure was calculated using several tools and methods. 748 amino acid sequences were obtained in FASTA format from Ensembl (https://asia.ensembl.org/index.html). Using SWISS 3D, comparative modeling was used to create the 3D structures of the normal and altered forms of OCA2. Figures reveal the family-06's normal and aberrant residues, Arginine replaces with Cyasteine at position 720 (Figure a& b) and Threonine with Methionine at Position 404 (Figure c & d).

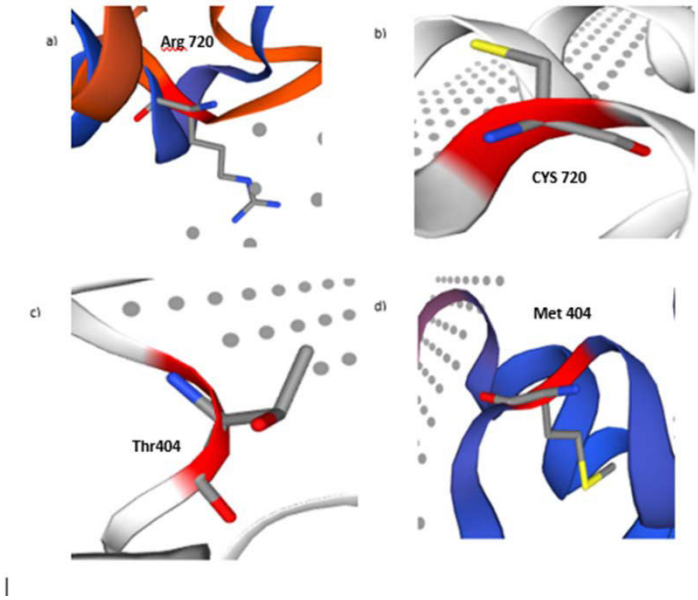

Figure 6OCA2 normal and mutant protein structure of IIU-OCA-10, first variant is p.Arg720Cys, a) is normal protein with Arginine at amino acid no 720, b) mutant protein with Cysteine residue at 720 amino acid, and second variant is p.Thr404Met, c) a normal pro

4.1.2.2 4.3.5.2 MLPH Gene

A variety of bioinformatics methods and software were used to determine the crystal structure of human MLPH. 600 amino acid sequences were obtained in FASTA format from Ensembl. Using SWISS 3D, comparative modeling was used to create the 3D structures of the MLPH normal and mutant structures. Figures show the modified and normal residues of IIU-OCA-10. Alanine stays the same after the mutation since the altered codon codes for Alanine, making it a silent mutation.(Figure 4.3.9 a).

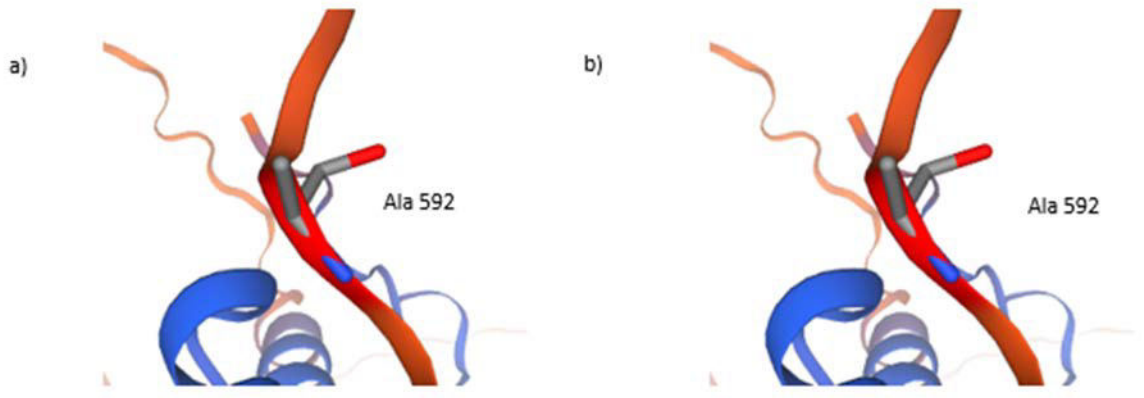

Figure 7Alanine remain same after mutation, because mutated codon codes for Alanine, so it is silnent mutation

4.2 OCA2 gene (IIU-OCA-08)

Mutation is in intronic region after exon 9, 15 base pairs upstream of exon 10, a single nucleotide variant (c.1045-15T>G) was discovered in the OCA2 gene. This mutation may interfere with proper pre-mRNA splicing since it is located near to the splice acceptor location of intron 9.

This mutation may change the splice acceptor recognition, cause exon skipping, or provide a cryptic splice site depending on its location in relation to the conventional acceptor site. Such disturbances may lead to frameshifted transcripts, intronic sequence inclusion, or exon 10 exclusion, all of which might ultimately impair the OCA2 protein's ability to function. Confirmation of the splicing impact of this mutation requires functional confirmation by minigene assays and in silico splice prediction techniques.

According to ACMG categorization standards, this variation is deemed probable harmful in clinical settings because of its capacity to interfere with vital splice site machinery.

4.2.1 Conservation study of OCA2 gene:

The conservation study of amino acid arginine at position 720 across multiple species shows that Arginine highly conserved at position 720 and mutation at position can bring significant consequences.

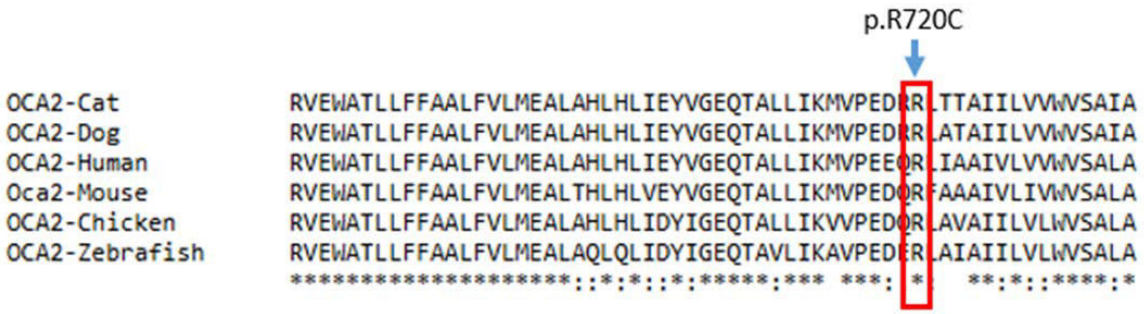

Figure 8 Arginine is highly conserved in this region

The conservation study of amino acid Threonine at position 404 across multiple species shows that Threonine highly conserved at position 404 and mutation at position can bring significant consequences.

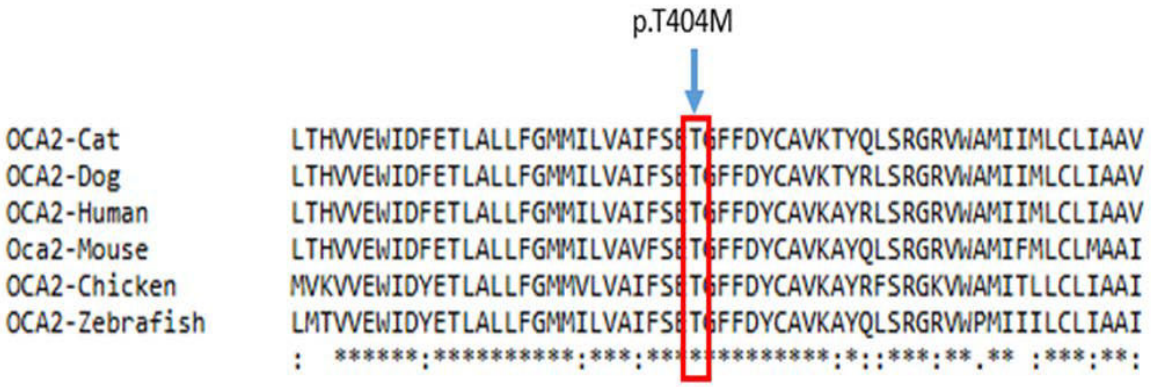

Figure 9 Threonine is highly conserved in this region

Another variant from IIU0OCA-08, the conservation study of amino acid is not possible because variant is in intronic region, which may alter splice site during post-transcriptional modification process which is splicing.

Discussion

Identifying possible genes and pathogenic variations that cause Oculocutaneous Albinism (OCA) in Pakistani families was the primary objective of the current study. In order to identify the underlying genetic causes, we specifically focused on finding families affected by OCA and doing whole exome sequencing (WES). We were able to identify causal variations in multiple families through thorough research.

The probands conducted whole exome sequencing, which was followed by extensive data validation and filtering. Interestingly, a reported missense and synonymous variant and novel intronic variant in the OCA2 gene was found in the families known as IIU-OCA-10 and IIU-OCA-08 respectively. After being further investigated using a variety of in silico prediction algorithms, this variant which had not previously been reported in any genomic databases was consistently predicted to be damaging. Its uniqueness and therapeutic relevance were validated through cross-referencing several online sources.

A protein that is essential to melanosome formation and a major factor in determining the generation of eumelanin in melanocytes is encoded by the OCA2 gene. By interacting with other proteins and adjusting melanosomal pH, it plays a critical role in controlling melanin synthesis and pigmentation (Mijke Visser, 2014). Oculocutaneous Albinism Type 2 (OCA2) is caused by mutations in OCA2 or total loss of its function. Additionally, tyrosinase and tyrosinase-related protein 1 (TYRP1) are trafficked and localized to the plasma membrane by the gene. OCA2 has long been regarded as a leading candidate to explain variations in human pigmentation due to its function in pigmentation and its placement inside the chromosome 15q11.2–q12 region. In the past, the p gene has also been linked to early-identified pigmentation abnormalities in mice. (Mohsin Shahzad, 2017)

The second leading form of albinism worldwide, including in Pakistan, is OCA2. This study discovered that affected Pakistani households had both known and unfamiliar OCA2 mutations. In a Pakhtoon family from Bannu, in the province of Khyber Pakhtunkhwa, a novel mutation (c.1234G>T; p.Val412Ala) was identified. Classical clinical features such as

white hair, white to reddish-white complexion, nystagmus, depigmented irides, and blurred vision were observed in affected individuals of these families.

Two other mutations that have previously altered a similar amino acid residue (p.Arg720Cys and p.Thr404Met) are reported in the literature in addition to the distinct variation. In a different family, we also discovered a similar variation (c.1776G>A; p.Ala592=). The OCA indicators appeared in all the families in the cohort. There might be regional founder mutations present because several of the variations observed in this study seemed to be prevalent among the Pakistani population.

Within intron 9, 15 base pairs upstream of exon 10, a unique intronic variation c.1045-15T>G was discovered in the OCA2 gene of family IIU-OCA-08. The canonical splice acceptor site, which is essential for precise exon identification during pre-mRNA processing, is located adjacent to this variation. Such placement raises the possibility of interfering with the regular splicing process, which might result in the inclusion of intronic sequences in the final transcript, activation of cryptic splice sites, or exon 10 skipping. Any of these consequences may cause an early stop codon or frameshift, which would impair the OCA2 gene product's functionality.

The behavior of comparable splice-altering mutations in other genes is known to support this assumption. For instance, over 98% of β-thalassemia cases are caused by four frequent mutations in the β-globin (HBB) gene, which are clearly indicative of splicing disruption in the Greek Cypriot community. These examples show how intronic mutations may change splice site fidelity through a range of processes, including as total exon skipping, the development of aberrant splice sites, and weakening of acceptor sites. The OCA2 c.1045-15T>G variant's potential pathogenicity may be inferred from its resemblance to several well-established splicing mutations in the absence of functional experiments.

To verify the impact of this mutation on transcript processing, more experimental minigene splicing tests and in silico splice prediction tools are required.

Table 7 shows how intron region varirants affect splice sites. (extracted from Book, Human Genome)

Diagram of a minigene assay where a strong promoter is used to clone the exon of interest and flanking intronic sequences into a splicing reporter vector. Total RNA is taken after the construct is transfected into cells that are capable of splicing. Primers from the vector backbone (arrows) are used in RT-PCR, and the results are examined by gel electrophoresis to determine if the splicing was proper or not. Intron 3 of the NF1 gene, which is five nucleotides downstream of the donor splice site, is an example of a G>C substitution. Exons and flanking intronic sections of both wild-type and mutant sequences were cloned into a minigene vector and transfected into HepB3 cells. The mutation caused exon 3 to be skipped, as demonstrated by RT-PCR and gel electrophoresis, indicating the disruption of normal splicing.(Baralle M, 2003)

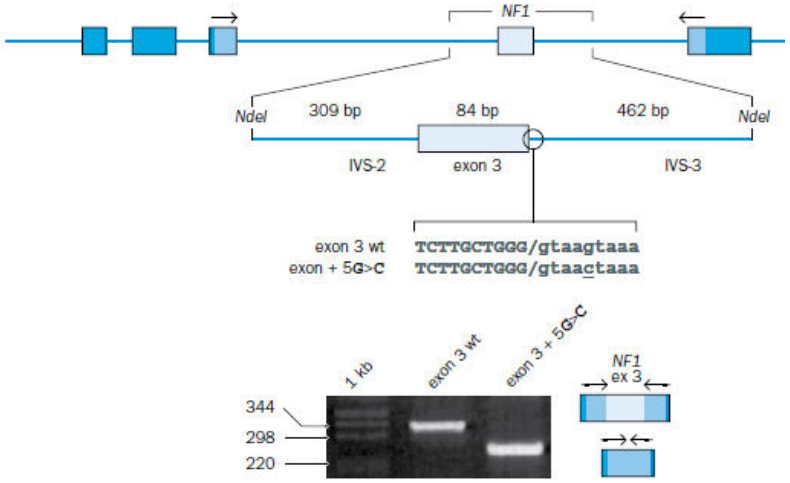

TABLE 16.3 β-GLOBIN MUTATIONS RESPONSIBLE FOR THALASSEMIA IN GREEK CYPRIOTS

Mutation	Location	% of all cases	Sequence change	Effect
c.92+1G>A	Intron 1	5.1	AGgttggtat AGattggtat	Abolishes a canonical gt donor site
c.92+6T>C	Intron 1	5.5	AGgttggtat AGgttggcat	Weakens a normal splice site
c.93–21G>A	Intron 1	79.8	ctattggtctattttccc ctattagTCTATTTTCCC	Activates a cryptic splice acceptor site in intron 1
c.316–106C>G	Intron 2	5.1	cagctaccat CAGgtaccat	Activates a cryptic splice donor site in intron 2

Figure 10 A minigene assay to assess splicing (Baralle M, 2003)

Variants in other genes, including MC1R or TYRP1, have been shown to alter OCA2 phenotypes, implying potential synergistic interactions across the pigment biosynthesis pathway. This demonstrates the complicated genetic makeup of OCA, where a number of genes may collaborate to influence the severity and appearance of the condition. (King Richard A., 2003) (Pei-Wen Chiang, 2008)

In the final analysis, this work improves our knowledge of the molecular spectrum of OCA in Pakistani people. The research results suggest that OCA is almost common this area, despite the absence of exact prevalence statistical data. The findings provide credibility to the emergence of more precise genetic testing and counseling methods for affected people and their families.

OCA has a very extensive genetic makeup that includes both intergenic interactions and unique gene effects. To clarify how these genetic variables, in conjunction with environmental factors, affect the clinical manifestation of OCA, more research is necessary. Deciphering the wider context of pigmentation abnormalities in varied groups will need a greater comprehension of these pathways.

Chapter No 05

Conclusion

Conclusion

This Research aimed to find the genotype-phenotype correlation for families affected with Oculocutaneous Albinism (OCA), having a particular focus on Pakistani families from the Khyber Pakhtunkhwa area. The need for better knowledge of OCA's genetic makeup, especially in populations with high consanguinity, is the driving force behind this endeavor. In order to evaluate the molecular foundation and phenotypic results in afflicted patients, the study included both genetic and clinical studies, including whole exome sequencing (WES) and detailed ophthalmological examinations.

Four OCA2 gene variations from two families were found by our study. A synonymous variation (c.1776G>A; p.Ala592=) and two missense mutations (c.2158C>T; p.Arg720Cys and c.1211C>T; p.Thr404Met) were discovered in a compound heterozygous state in one family. Even these synonymous mutation is frequently regarded as benign, their co-occurrence with harmful missense mutations suggests that they may play a role in splicing or regulatory processes, which calls for more functional research. The pathogenic potential of the two missense variations is supported by the fact that they impact evolutionarily conserved amino acid residues and were predicted to be harmful by many in silico methods.

An intronic region mutation (c.1045-15T>G) was found in the second family (IIU-OCA-08). This variation is likely to be pathogenic and is found in a non-coding region of the OCA2 gene. It has already been previously described in public databases. So it is pathogenic and have effect on protein structure because this region is altered during splicing.

Every afflicted person from both families displayed the typical OCA phenotype. Clinical signs and symptoms included photophobia, nystagmus, pale complexion, and white or very light-colored hair. There were noticeable visual complaints, and everyone reported having less visual acuity. Foveal hypoplasia and a hypopigmented albinotic fundus, two characteristics of OCA that are consistent with the pathogenic variations found, were found during ophthalmological tests. These clinical results confirm the influence of the noted genetic alterations on pigmentation and ocular development and support the genotype-phenotype relationship.

This study significantly expands understanding of OCA2-related mutations and the phenotypic effects they have on the Pakistani community. The hypothesis that OCA2 plays a significant role in OCA in this area is reinforced by the discovery of both known and new variants. Furthermore, the complicated inheritance patterns that can arise even in consanguineous families are highlighted by the discovery of compound heterozygosity among afflicted people.

This study significantly expands understanding of OCA2-related mutations and the phenotypic effects they have on the Pakistani community. The hypothesis that OCA2 plays a significant role in OCA in this area is reinforced by the discovery of both known and new variants. Furthermore, the complicated inheritance patterns that can arise even in consanguineous families are highlighted by the discovery of compound heterozygosity among afflicted people.

Discovering the causal variations is only one aspect of this study's importance; another is its wider ramifications for Pakistani healthcare and genetic counseling. Better therapeutic care, including photoprotection techniques and visual rehabilitation, can be facilitated by an early molecular identification of OCA. Through prenatal diagnosis and carrier screening, it can also help guide reproductive decisions, especially in areas where consanguineous marriages are prevalent. Clinicians and geneticists can forecast illness severity, offer precise prognoses, and direct suitable therapies by establishing genotype-phenotype correlations.

Furthermore, this work gives useful information to worldwide databases and advances the global effort to catalog mutations linked to albinism. The discovery of a new variation stimulates additional population-based and functional research to ascertain its prevalence and harmful causes. It also emphasizes how important it is to create localized genetic panels for albinism that accurately represent the range of mutations seen in the Pakistani community.

Moving forward, the discovery of more uncommon or non-coding variations that could contribute to OCA will be made possible by the incorporation of next-generation sequencing (NGS) technologies, such as whole genome sequencing (WGS). Furthermore, integrating transcriptomic, environmental, and epigenetic data with genetic data may provide a greater understanding of the variability and development of illness.

In conclusion, this study demonstrates that clinical characteristics correlate molecular findings and highlights the value of genetic studies in comprehending hereditary pigmentation diseases. In order to assist impacted people and their families, it also emphasizes the necessity of funding genetic research, testing, and counseling services throughout Pakistan. Population-specific research like this one will be crucial in determining the direction of tailored medical care and focused treatment approaches for hereditary illnesses like OCA as precision medicine develops.

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