Mutational analysis of exons 6 and 7 of the *MYO7A* gene in consanguineous Usher syndrome affected families from Sindh.

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2023

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

In

MOLECULAR BIOLOGY

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2023

بسم الله الرحمن الرحيم

DECLARATION

I hereby declare that the material and information presented in this thesis in my original work. I have not previously presented any part of this work, **"Mutational analysis of exons 6 and 7 of the *MYO7A* gene in consanguineous Usher syndrome affected families from Sindh."**

Shehzeen Fatima

Dedication

***Them that I love know that I love them*.**

To my grandfather, Glulam Murtaza, this is dedicated to him, him first, for being my one-man army and my grandmother who wrapped me in her warmth to last forever.

Then to my mother for her untiring efforts to let us witness these days and my innocent father whose absence echoes like a void that shadows everything.

And last, to my incredible brothers for their unshakable trust and financial support.

ACKNOWLEDGEMENT

Gratitude to the Omnipresent, who let it be and it became!

I would like to express my heartiest gratitude to my supervisor Dr. Sabika Firasat for her guidance, unbiased supervision, and, most importantly, for keeping my mental health unharmed.

I extend my sincerest appreciation to the Chairperson Dr. Amina Zubairi whose leadership and dedication have steered the department to success.

Bundle of thanks to the families that cooperated in my research.
I am highly indebted to Dr. Saleh Memon from AL-Ibrahim Trust Eye Hospital, Karachi, for being so generous and kind to his extraordinarily well-mannered staff for their cooperation.

I also thank LRBT Hospital, Karachi, for their support.
I pay my cordial gratitude to Dr. Aamir Memon for his assistance, and Dr. Shazia Aftab, and Aftab Bijarani for building me paths against all odds throughout the sampling time.

Great thanks to my seniors, Raeesa, Alisha, Tayyaba, and Zainab for always helping and guiding the best.

My deepest gratitude to my family: my lovely grandparents, beloved father praying from heaven, selfless mother, and four incredible brothers for the gravitational pull to keep me grounded.

To the savior friends, Areej Mushtaq, Noumana Malik, and SafiUllah Khan, who emotionally and morally helped me turn my blood sweat, and tears into this thesis, I pay them my heartiest gratitude.

Last, to my roommates, Tehniat Zafar and Maheen Khalid, who made sure that I never quit, I owe them gratitude for making me feel at home miles away from home.

List of Abbreviations

AE	Auto acoustic emission
ASO	Antisense oligonucleotide
BR	Auditory-brainstem response
ASOs	Short synthetic modified nucleic acid
ASSR	Auditory stead-state and stapedial reflexes
AVV	Adeno-associated virus
CDH	Cadherin
DNA	Deoxyribonucleic
DFNA	Autosomal dominant sensorineural hearing loss
EBr	Ethidium bromide
IHC	Inner hair cell
MYO7A	Myosin 7A
OHC	Outer hair cell
PK	Proteinase Kinase
PCR	Polymerase Chain Reaction
RP	Retinitis Pigmentosa
SDS	Sodium Dodecyl Sulphate
EPR	Retinal pigment Epithelium
TRIDs	Transitional read-through-inducing-drugs
TBE	Tris-borate EDTA
USH	Usher syndrome
USHI	Usher syndrome type 1
USH2	Usher syndrome type 2
USH3	Usher syndrome type 3
UV	Ultraviolet
VHC	Vestibule hair cell

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ABSTRACT

Usher syndrome causes simultaneous loss of hearing and vision as well as vestibular dysfunction in some, but not all cases. It is the most common cause of deaf-blindness globally, with a prevalence of 4 to 17 per 100,000 individuals. It is inherited in an autosomal recessive manner. USH is classified into three clinical types: USH1, USH2, and USH3. Each of these types is further divided into 14 subtypes in total, brought on by alterations in distinct genes. Nine genes have been identified with accuracy causing the onset of USH: including *MYO7A, USH1C, CDH23,PCDH15, USH1G* (S*ANS* for USH2A), *ADGRV1, WHRN*, and *CLRN1*. Many of the genes involved in USH are also found in non-syndromic RP and non-syndromic hearing variants, demonstrating the high genetic diversity of this condition. *MYO7A* gene is one of the most involved genes in the case of Usher syndrome and is located on chromosome 11q. It belongs to the superfamily of motor genes, reporting 70% of USH1B cases when mutated, causing severe to profound hearing loss, RP, and vestibular areflexia. Due to insufficient sources of diagnosis and rehabilitation, the prevalence rate of USH isn't documented comprehensively on a global level. According to numerous studies, consanguineous marriages frequently result in the transmission of autosomal recessive traits, and Pakistan is one of the countries where such marriages are relatively common. In this study, we selected 4 USH-affected families from Sindh, Pakistan, having a positive family history with consanguineous marriages practiced commonly. Blood samples were collected from the hospital. DNA from the blood sample was extracted in the lab of Molecular Biology, Quaid I Azam University, Islamabad. Followed by gel electrophoresis, PCR was run to amplify exons 6 and 7 of the *MYO7A* gene of probands from each of the 4 selected families. Sanger's sequencing of purified PCR products showed a disease-causing variant **c.640G>A** in exon 7 of *MYO7A* in a clinically diagnosed USH patient in one of our 4 families, having congenital hearing loss, progressive RP, and vestibular areflexia, proving exon 7 as one of the hotspots in Pakistani population causing USH1B. In-silico analysis of c.640G>A highlights that this disease-causing variant changes the splice site, interfering with proper protein features and halting it from performing its function correctly, which could lead to the development of USH. However, the other 3 families do not exhibit any sequence variation in the targeted *MYO7A* exons, emphasizing the need for further sequencing. For future perspective, genetic counseling must be given to avoid consanguineous marriages and as the disease is highly heterogeneous, better innovations like whole genome sequencing should be done.

CHAPTER 1
INTRODUCTION

1. INTRODUCTION

Usher syndrome is a medical condition of double sense impairment causing deaf-blindness, which limits the participation of an individual in society and the ability to exist with freedom and security (*Castiglione et al.,2022*). In 1858, Albrecht von Graefe first described this, however, the name of the syndrome dates to ophthalmologist, Charles Usher who introduced 40 families comprising 69 patients who were affected by both hearing loss and retinopathy (*Usher et al.,1914*). This genetic disorder involves instances of hearing impairment, retinopathy, and vestibular areflexia, characterized by various onsets and manifestations. (*koenekoop et al.,1993; Reineers et al.,2006*). It is transmitted via autosomal recessive inheritance *(Josarra et al.,2010)***.** USH is categorized into 3 types based on clinical profile and 14 subtypes based on the kind of mutations involved in different genes and loci. (*Qu et al., 2017; Toms et al.,2020).* Even though several genes are implicated in some way in causing the syndrome, nine genes have been confirmed to cause the three subtypes of Usher syndrome to date. The genes are: *MYO7A, USH1C, CDH23, PCDH15, SANS, USH2A, ADGRV1 WHRN and CLRN1* (*Catiglione et al.*,*2022*).

As the genes and proteins involved in the prognosis of Usher syndrome impair the ciliary functions of cells, ciliopathy is commonly used to describe this condition (*Tsang et al., 2018*; *Fuster et al., 2021*). Most genetic mutations that cause the syndrome result in the disruption of structural proteins in the auditory, visual, and vestibular systems. However, the issue is still debatable *(Tanimoto et al. 2011)*. In the case of USH, the sensory cells that express actin filaments at specific locations are observed to be constrained, which explains why the eyes and ears are particularly affected by the impairment (*Chaib et al.,1997*).

1.1. Anatomy and function of sensory organs targeted by Usher syndrome.

The inner eye and ear are the main target organs in Usher syndrome. *(Moler et al.,2022).* As per the framework of USH, the seriousness of clinical symptoms is contingent upon the degree of sensory impairment in these two organs *(Sedigheh et al.,2022).*

1.1.1. The inner ear and the sensory hair cells

Sensory organs for balance and hearing are the vestibule and cochlea; both of which are present in the inner ear *(Delmaghani et al.,2020).* Mechanoelectrical transduction facilitates hearing and balance. The transduction occurs in hair cells that are highly specialized, polarized epithelial cells: type I and type II in the vestibule (VHCs) and inner and outer hair cells in the cochlea (IHC and OHC). The hair bundle constitutes the mechanosensitive organelle found within all the hair cells. Situated a short distance above the apical surface of the cell, projects 50-100 F-actin-filled stereocilia, constructing a staircase pattern increasing toward- kinocilium that is short-lived during cochlear sensory hair cells' development (*Delmaghani et al.,2020).*

The hair bundle's organization, geometry, and physical and functional properties differ according to the hair cells' position in sensory organs. However, the basic principles of function remain the same. Consisting of 4-5 rows of stereocilia forming a staircase, the hair bundles of the vestibule are compact. The OHC hair bundles in the cochlea are arranged in 3 rows forming a staircase of V or W shape, whereas IHC hair bundles form U shape due to flatter, curved profile. The cochlear hair bundles convert sound-induced displacements in stereocilia (even in nanometers) into membrane potential changes, releasing neurotransmitters in IHCs and transmitting signals to the brain (*Delmaghani et al.,2022).*

Figure 1.1 illustrates the anatomical structure of the mammalian ear, namely type I and type II, specialized in sensing the motion of the head and body within the vestibule. Within the cochlea, auditory perception takes place through the interaction of sound waves with two distinct sets of hair cells: the inner hair cells (IHCs), pivotal in transmitting auditory signals to the brain, and the outer hair cells (OHCs), which function as mechanical amplifiers. These outer hair cells enhance the cochlea's sensitivity and enable it to discriminate between various frequencies effectively. *(Delmanghani et al.,2020).*

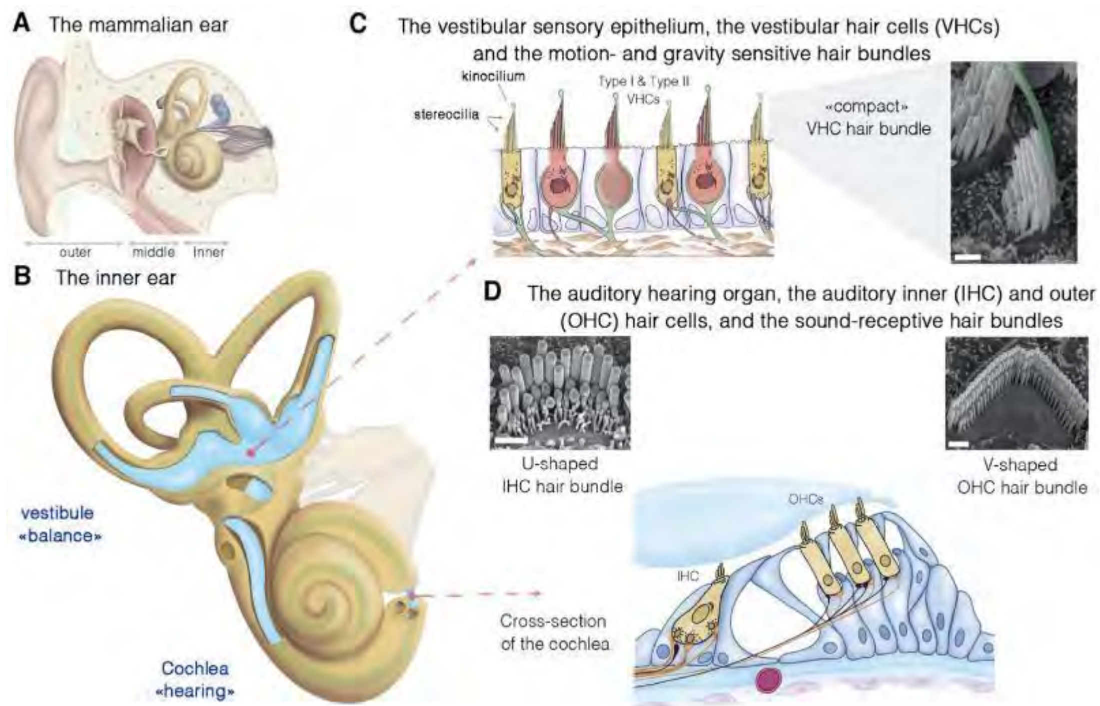

Fig 1.1. Anatomy of the ear in mammals **A**: The 3 main compartments of the mammalian ear are the outer middle, and inner ear. **B**: Vestibule (balancing organ) and the cochlea (hearing organ) of the inner ear. **C, D:** The hair cells, accompanying innervation, and numerous types of supporting cells make up the vestibular (C) and auditory (D) sensory epithelia.

1.1.2. Retina and the photoreceptor cells

Covering the rear portion of the eye, the retina constitutes a neural stratum housing photoreceptor cells alongside retinal pigment epithelium cells (RPE). Before reaching the photoreceptor cells, which are rods and cones, light needs to traverse a sequence of structures: cornea, lens, vitreous, and retinal cells *(Boto et al., 2012; Crane et al., 2021*). In the outer segment of retinal cells, that is the site of phototransduction. The visual signal is initiated by opsins, that trigger the cascade *(Crane et al.,2021*).

Figure 1.2 illustrates the eye's anatomical structure, displaying the retinal layers that encompass RPE cells, alongside rod and cone photoreceptor cells. These photoreceptors are interconnected with horizontal cells, bipolar cells, amacrine cells, and ganglion cells within the inner retina. Additionally, Müller cells, which are glial cells, extend across all layers of retinal cells.

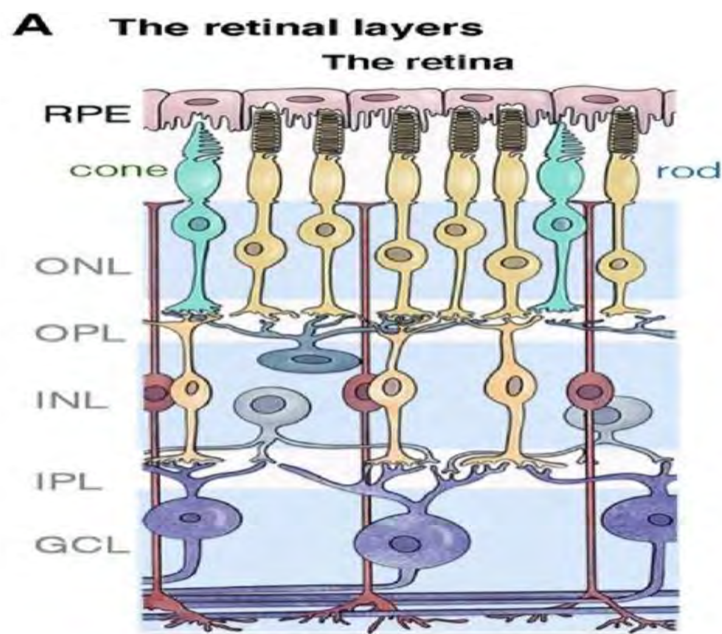

Figure 1.2. A: Illustrating the layers of the eye's anatomy that comprise the retina: The retinal pigment epithelial (RPE) layer, the outer nuclear layer (ONL), the inner nuclear layer (INL), the ganglion cell layer (GCL), the outer plexiform layer (OPL), and the inner plexiform layer (IPL).

1.2. Classification of Usher syndrome

A simultaneous display of bilateral sensorineural hearing impairment and retinitis pigmentosa, which is a degenerative condition affecting the retina, occurs in this condition. Based on the extent and timing of hearing loss as well as the existence or lack of vestibular dysfunction, Usher syndrome is categorized into 3 clinical subtypes. Within each subtype is seen variability, overlapping, and atypical presentation. (*Cohen et al.,2007).*

1.2.1 Usher syndrome type 1 (USH I)

It is the most serious among the three clinical subtypes. Accounts for 25-44% of all the syndromic cases (*Reiners et al.,2006).* Severe to profound bilateral congenital sensorineural deafness along with non-progressive, vestibular areflexia occurs in USH1-affected individuals. Vestibular areflexia is compensated to a certain level in older age using vision, until the outbreak of retinitis pigmentosa. (*Toms et al.,2020*). Hearing aids are not of much use in this case, due to the severity of deafness. However, cochlear implants on time could establish oral communication and open the perception of speech. *(Pennings et al.,2006; Jatana et al.,2013)*

1.2.2. Usher syndrome type 2 (USH II)

It is the most common among the three clinical subtypes of Usher syndrome, accounting for almost half of the cases *(Reiners et al.,2006*). The sensorineural hearing loss in this case is represented as sloping, that is: in low frequencies, it is mild to moderate, and in sound of higher frequencies, severe to profound (*Abadie et al.,2012*). Conventional hearing aids are found helpful for the affected children and have almost normal speech. Vestibular functioning in this subtype is unimpaired with some exceptions in a few confirmed cases *(Magliulo et al.,2017).*

1.2.3. Usher type 3 (USH 3)

It is the rarest one among the three clinical types of Usher syndrome. It constitutes approximately 2-4% of total cases, with a higher occurrence among the population of Finland. (*Pakearinean et al.,1995)* and Ashkenazi Jewish *(Ness et al.,2003).* Hearing loss is usually detected in the first decade and can also be delayed. The nature of hearing is progressive. Roughly 50% of the patients display vestibular irregularities, although the majority indicate a typical age for commencing independent walking. During the initial phases, hearing aids prove advantageous, but if hearing loss advances, cochlear implants could become necessary. (*Sadeghi et al., 2005).*

In all the types, audiological findings are present before the ophthalmological ones. RP develops in each type, but the onset varies; pre-adolescent in the case of type 1, in the first two decades of life in the case of type 2, and post-pubertal in the case of type 3 *(EI-Amraoui et al., 2014).*

Table 1.1 shows the summarized clinical features with associated genes in 3 subtypes of Usher syndrome *(Toms et al.2022)*.

Table 1.1. Clinical features and associated genes in USH subtypes

Usher subtypes	Causative genes	Sensorineural Hearing loss	Retinitis Pigmentosa	Vestibular Function
Usher 1	*MYO7A* *USH1C* *CDH23* *PCDH15* *USH1G* *CIB2*	Congenital, Severe to profound	Onset: Prepubertal The average age of diagnosis: 2nd decade	Hypofunction. Motor development is delayed. Infants do not walk till 18 years of age
Usher 2	*USH 2A* *ADGRV1* *WHRN*	Congenital, Moderate to severe	Onset: 2nd decade The average age of diagnosis: 3rd decade	Normal
Usher 3	*CLRN1*	Post-lingual onset. progressive	Variable onset, most often in 2nd decade	Variable; usually mild abnormalities in 50% of patients

1.3. Clinical presentation of patients with Usher syndrome

Usher syndrome is marked by bilateral sensorineural hearing loss and progressive retinal degeneration manifested as RP. The severity and onset of hearing loss, as well as the presence of vestibular dysfunction, determine the type of USH present. Clinical heterogeneity exists within each subtype of Usher, with overlapping and atypical presentations *(Cohen et al.,2007).*

1.4. Diagnosis

USH is diagnosed based on hearing loss and RP, with the former being congenital and the latter manifesting primarily in childhood and adolescence. In most industrialized nations, clinical diagnosis precedes genetic diagnosis to ensure accuracy. After this, next-generation sequencing is employed to target all identified genes associated with each variant of Usher syndrome *(Toms et al.,2020).*

1.4.1. Hearing Loss

Hearing loss in all types of Usher syndrome is congenital. Otoacoustic emissions (OAE), auditory-brainstem responses (ABR) using threshold measurements, auditory steady-state responses, and assessments of stapedial reflexes (ASSR) are employed to ascertain the extent of hearing impairment and auditory neuropathy in newborns. Hearing loss is confined to the cochlea in USH, where both inner and outer hair cells are eliminated. Hearing impairment differs across all subtypes of USH *(Castiglione et al.,2022).*

USH1: The hearing loss is congenital, and deafness is bilaterally profound (*Castiglione et al.,2022).*

USH2: Congenital hearing loss typically manifests as moderate to severe bilateral impairment. Typically, the initial three to four decades of life are characterized by consistenthearing levels. Auditory degradation commonly exhibits a sloping pattern, primarily ranging from mild to moderate in lower-frequency auditory ranges and progressing to a severe to profound extent in higher-frequency ranges (*Castiglione et al.,2022).*

USH3: Hearing loss is congenital. In childhood, it is typically mild. The condition tends to advance over time, and individuals usually experience significant hearing loss ranging from severe to profound during their third decade of life, making it distinct and more variable than USH2 *(Castiglione et al.,2022).*

1.4.2. Balance

USH1: All children with USH1 demonstrate bilateral vestibular areflexia, hypotonia, or delay in sitting and crawling, which is associated with delayed gross motor development in neonates. Later, these infants have difficulty when it comes to walking on balance beams and acquiring the skills to ride a bicycle *(Castiglione et al.,2022).*

USH2: Normal vestibular function is present in patients with USH2. *(Castiglione et al.,2022).*

USH3: During childhood and early adulthood**,** the vestibular function is mostly normal. However, it decreases later in adulthood (*Castiglione et al.,2022*).

1.4.3. Vision loss

Retinitis Pigmentosa is the cause behind retinal degeneration that eventually leads to vision loss. In the case of Usher, RP isn't comparable with its other forms, as in the case of USH, it progresses very slowly. It involves degenerating two types of photoreceptor cells: rods and cones. The rods help and function in night vision and are in the periphery of the retina, aiding in peripheral vision. Whereas cones are responsible for day, central, and color vision. In childhood, vision loss is mild, and often undetected for the first decade of life. Sensitivity towards light increases gradually as rods start degenerating (*Malm et al.,2011*). The degeneration of rods increases in young adulthood, causing tunnel vision. In most cases, by the age of 30-40 years, cataracts develop. In the case of USH, several people have central vision preserved throughout their lives, with a visual field of 5-10 degrees (*Kimberling et al.1995; Sadeghi et al.2006).* Figure 1.3 shows the fundus examination of patients affected with Usher syndrome. Boney spicules, pigmentation, and attenuated vessels clearly show the symptoms of retinitis pigmentosa.

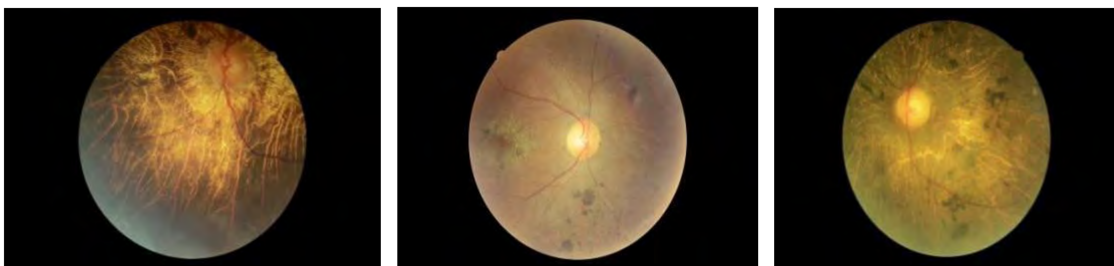

Fig1.3. Fundus photograph of patients with Usher syndrome.

1.5. Epidemiology

In the general population, USH is a rare condition (*Toms et al.,2020).* However, for professionals, ophthalmologists, audiologists, otolaryngologists, and geneticists, it is considered as a common condition. Due to the prevalence of mutations responsible for Usher syndrome, it stands as the primary contributor to both ciliopathies and the condition of deaf-blindness *(Castiglione et al.,2022*). The prevalence of USH is not adequately described globally. Huge variations have been reported, likely attributable to a lack of rehabilitation resources and improper diagnosis *(Castiglione et al.,2021).* Britain, Sweden, Norway, Finland, Denmark, the Netherlands, the United States, and African nations have accurately reported the prevalence (*Espinos et al., 1998*), as have Sweden, Norway, Finland, and Denmark. In contrast, only limited data has been gathered from Asia and Africa; nevertheless, prevalence estimates have been derived for many countries and ethnic populations within these continents. (*Castiglione et al.,2022*). In general, the worldwide occurrence rate of Usher syndrome (USH) is approximately 1 in 30,000 individuals. This figure should be considered as a moderate average since different research studies have indicated a wide spectrum of prevalence, spanning from 1 to 17 cases per 100,000 people. *(Espinos et al.,1998).* The prevalence of 10/100,000 people has been reported after clinical and genetic confirmation by A Swedish USH database managed by theauthors *(C. Moller personal communication*). In children, congenital profound deafness attributed to USH constitutes a percentage of 9.2 *(Kimberling et al.,2010).* Therefore, USH isthe most common cause of syndromic hearing loss after Pended syndrome. There is great variety in the prevalence of different subtypes of USH and the mutations that cause the condition (*Castiglione et al.,2022).* The most common form, holding 60 % is type 2 of Usher syndrome (USH2), in which the mutation in the USH2A gene accounts for 80-90% of the cases *(Colombo et al.,2021*). In the case of USH1, there has been reported great variety and heterogeneity among different countries and ethnicities. In contrast, USH3 is uncommon on a global scale, except in Finland, where the occurrence of USH1 stands at 34%, USH2 at 12%, and, notably, USH3 at nearly 40%. *(Vastinsalo et al.,2013*). Usher syndrome, observed among Ashkenazi Jewish affected families, serves as an illustration of an ethnic isolate. Within spectrum of Usher syndrome cases, it's noteworthy that Usher syndrome type 3 (USH3) constitutes a significant proportion, approximately 40%. (*Ness et al.,2003).*

Table 1.2 highlights the types of each of the 3 USH types with the prevalence of mutation caused in distinct genes.

Table 1.2. USH types and subtypes with prevalence as per mutations.

USH TYPE 1 (35-40%)	Gene	Protein	Epidemiology (% mutation)	Year of Identification
USHIB	*MYO7A*	Myosin VIIA	50-70%	1995
USHIC	*USH1C*	Harmonin	6-20%	2000
USHID	*CDH23*	Cadherin23	0-20%	2001
USHIF	*PCDH15*	Procadherin15	5-10%	2001
USHIG	*USH1G*	USH type1G protein	0-5%	2003
USH Type 2 (60-65%)	**Gene**	**Protein**	**Epidemiology (% mutation)**	**Year of Identification**
USHIIA	*USH2A*	Usherin	50-80%	1998
USHIIC	*ADGRVI*		5-20%	2994
USHID	*WHRN*	Whirlin	0-10%	2007

Usher type III (0-5%)	Gene	Protein	Epidemiology (% mutation)	Year of Identification
USHIIIA	CLRN1	Clarin-1 Transcript variant	90-95%	2001

1.5.1. In Pakistan

Pakistan is among the countries where consanguineous marriages are prevalent, reportedly 70% (*Ullah et al., 2018).* Many such marriages result in the passing of autosomal recessive traits (*Coellner et al.,2018*). Due to increased homozygosity by descent, the offspring of consanguineous couples are at higher risk of autosomal recessive disorders, just like UHS. *(Coellner et al.,2018*). As far as the prevalence of USH in Pakistan, there is no comprehensive data. However, there are case reports of Usher syndrome from Lahore, Karachi, Rawalpindi, and KPK (*Trop et al.,1995; Afzal et al.,2009; Awan et al.,2020).*

Table1.3 shows Usher syndrome types and subtypes caused by the confirmed reported genes and their prevalence in the general population, determined by epidemiological studies *(Castiglione et al., 2022).*

1.6. Inheritance pattern of USH

All the types of Usher syndrome are inherited in an autosomal recessive fashion; that is, both parents must carry the mutation, and it can cause disease when both mutant copies arrive in offspring *(Yan et al., 2010)*. When the parents are asymptomatic carriers of the usher gene, with each pregnancy, 25% of offspring will likely be born with USH. *(Josarra et al.,2010)***.**

Figure 1.4 shows the autosomal recessive inheritance pattern in USH, where both the parents are asymptomatic carriers of the disease and one offspring out of four is affected with the disease, one normal and two carriers. *(Josarra et al.,2011).*

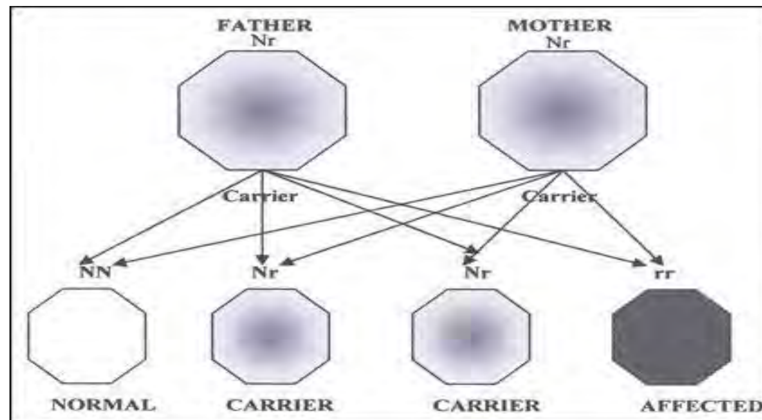

Figure1.4. An autosomal recessive pattern of inheritance in USH

1.7. The genetic landscape of Usher syndrome

The mode of inheritance of all types of Usher syndrome is autosomal recessive *(Yan et al.,2010)*At least 10 causative genes have been identified causing the disease *(Bonnet et al.,2011).*In usher type 1 there are nine loci known to be involved with identified gene*s: MYO7A* causing USH1B (*Well et al.,1999*), *USH1C;* USC1C *(Verpy et al.,2000), CDH23*; USH1D (*Bolz et al.,2001*), *PCDH15*; USH1F (*Ahmed et al.,2001*) *USIG*; USHIG (*Weil et al.,2003*) and *CIB2*; USHIJ (*Riazuddin et al.,2012*). The most frequent gene causing USH1 is *MYO7A*, accounting for more than half of the cases (*Stabeij et al.,2012)*. In the case of Usher type 2, three genes have been identified causing the disorder when mutated. The genes are *USH2A* causing subtype USH2A (*Eudy et al.,1998*), *ADGRV1;* subtype USH2C *(Weston et al.,2004*), and *WHRN* causing USH2D (*Ebermann et al.,2007*). Currently, only one gene, when mutated, has been confirmed to cause type III of Usher syndrome, *CLRN1* or (*USH3A) (Joensuu et al.,2001; Adato et al.,2001)*. Several structurally and functionally different proteins are encoded by usher genes, including an actin-binding motor protein; *MYO7A* (*Weil et al.,1995*), cell adhesion proteins; cadherin 23, protocadherin 15 (*Ahmed et al., 2001*), scaffolding proteins; Harmonin (*Veprey et al.2001*), usherin *(Eudy et al., 1998*), etc. In a wide range of tissues, most of these proteins are manifested in various splice forms and variants *(Wijik et al., 20014; Bhattacharya et al.,2002*), however, within the inner ear and retina, the entirety of usher proteins can be found, constructing formations that are limited to specific subcellular regions

namely, the inner ear's hair cells and the retina's photoreceptor cells (*Toms et al.,2015; Geleoc et al.,2020).* The involvement of usher proteins in functions like protein and organelle transport, mechano-transduction, cohesion, and synaptic maturation are indicated by different research studies (*Toms et al.,2020).*

1.8. Genotype-phenotype correlation

Diversity among usher genes is significant, and the different mutations they undergo have been associated with non-syndromic conditions such as autosomal recessive retinitis pigmentosa (RP) or autosomal dominant/recessive sensorineural hearing loss (known as DFNA or DFNB) (*Khan et al.,2011; Riazuddin et al.,2012*; *Ebermann et al. 2017*). Genetic variations within the *MYO7A* gene have been documented concerning recessive and dominant forms of non-syndromic hearing loss (*Liu et al.,1997; Weil et al.,1997).* It has been known that when a mutation allows some unconsumed protein motor function, for example, in-frame deletion c.5146_5148delGAG p. (Glu1716del), it gives rise to milder non-syndromic phenotypes. In contrast, the effect on protein function is more severe in the case of usher 1-associated mutations, as seen in the case of frame deletion c.1309G > A p. (Asp437Asn) (*Riazuddin et al.,2008).* A specific analysis of 33 individuals diagnosed with USH1B revealed that stop mutations located in the coding segments of the motor domain in *MYO7A* alleles, like the mutation c.999T.C p. (Tyr333*), might lead to less severe visual impairment compared to missense variants. This distinction is attributed to the absence of the mutated protein in stop mutations preventing the development of disease-related abnormalities *(Jacobson et al.,2011).* In families of Chinese origin where both Usher 1 and Usher 2 phenotypes are present, instances of *MYO7A* mutations have also been documented as a potential cause for the manifestation of auditory neuropathy within the phenotype (*Wrong et al.,2014; Xia et al.,2017).*

The comparable mutations in the *MYO7A* gene reported to cause DFNB2, as well as Usher 1, separately generate the question of whether the missed RP results in phenotype or whether there could be certain factors responsible for modification that eventually led to the phenotype *(Astuto et al.,2002).*

1.9. Physical and psychological health of Usher patients

In recent research, when contrasted with a representative sample of individuals from Sweden, the patients of Usher syndrome have shown poor physical health, encompassing symptoms like fatigue, headache, most prominently neck and shoulder, as well as psychological impairment *(Ehn et al.,2018)*. Patients showed a lack of social trust, an inability to problem manage, and a statistical over-representation of suicidal behaviors *(Ehn et al., 2018; Wahlqwist et al.,2020)*. Thus, the psychosocial consequences of deaf-blindness caused by Usher syndrome must not be neglected. Rehabilitation, including family, educational institutes, workplaces, etc., must be focused on *(Castiglione et al., 2022)*.

1.10. Ultra rare USH genes

These are the genes associated with Usher syndrome that has been reported to result in phenotypes that are inconsistent with the defined clinical subtypes; therefore, they are referred to as ultra-rare USH genes *(Nolen et al., 2020)* including *PDZD7 (Esisenbereger et al.,2012), ABHD12 (Esinberger et al.,2012), HARS (Puffenberger et al.,2012), CIB2 (Riazuddin et al., 2012) CEP78 (Numburi et al., 2016), CEP250 (Khateb et al.,2015), ESPN (Ahmed et al.,2018) and ARSG (Khuteb et al.,2018).*

1.11. Treatment

Currently, there is no available cure that could eradicate the condition or a safer treatment that works for each gene size and mutation *(Toms et al.,2020)*. However, several therapeutic strategies are under development for the retinal damage caused by USH since congenital hearing loss persists from birth and typically endures throughout one's lifetime. *(Geleoc et al.,2020)*. The strategies include gene replacement, gene editing, antisense oligonucleotide (ASO), and nonsense suppression *(Toms et al.,2020)*. In most therapeutic studies, patient-derived cells (fibroblast) or mutant mice's cell lines have been used *(Toms et al.,2015; Geleoc et al.,2020)*.

1.11.1. **Gene Replacement**: The technique involves the introduction of exogenous nucleic acid through vectors in place of the mutated gene within a living system to reverse the genetic defect *(Prasoon et al.,2018).* This strategy has demonstrated its efficacy in various Usher mouse models. Adeno-associated virus vectors (AAV) have been employed for the genes *MYO7A*, Whrn, and Clrn (*Colella et al.,2013; Dyka et al., 2014; Zallochi et al.,2014; Denulescu et al., 2016).* Functional *MYO7A* gene to USH1B mouse model retina has also been delivered by lentiviral-based vectors with large carrying capacities that proved successful. *(Hashimoto et al.,2007; Zalochi et al., 2014).* In mouse models of *USH1C,1G, 2D, H3, and* AAV vectors have also proved notable improvement in auditory and vestibular hair cell function *(Pan et al.,2017; Emptoz et al.,2017; Isgrig et al., 2017 132; Geng et al., 2017; Lu B et al., 2010).* This approach has an advantage: the viral DNA used is non-pathogenic to humans, where it has shown long-term transduction after a single induction, and no harmful side effects have been observed. For the large USH genes, limited cargo capacity could be a potential disadvantage *(Alessandro et al.,2020).*

1.11.2. **Gene Editing***:* In this technique, by using a nuclease enzyme, a DNA error is corrected through homologous recombination using a DNA template with a wild-type sequence *(Li et al.,2014).* This approach is appropriate for genes of any size and could be used to correct point mutations, splice mutations, and small indels. (*Toms et al.,2020).* Recently, the CRISPR/Cas9 system has been highly regarded and even won the Nobel Prize in 2020 *(Toms et al.,2020).* This allows in vitro correction of the c2299delG mutation in the USH2A gene through genetic modification *(Fuster et al.,2017).* For the treatment of IRDs, the CRISPR/Cas9-based technique shows promising results *(Toms et al.,2020).*

1.11.3. **Transitional read-through-inducing drugs (TRIDs):** It involves using small molecules to treat USH. The TRIDs bind to translational machinery, locating premature stop codons and inserting amino acids so that nonsense mutations can be read through (Toms and others, 2020). Several TRIDs have been used in vitro, in cell cultures, and in retinal explants to suppress Usher1-associated PCDH15 and USH1C mutations. *(Rebibi et al.,2007; Goldmann et al.,2010; Nudelman et al.,2010; Goldmann et al.,2012).*

1.11.4. Short synthetic modified nucleic acids (ASOs): These compounds connect with RNA through complementary base pairing *(Toms et al., 2020).* It is feasible to modify these compounds to enable them to attach to specific regions within pre-mRNA, such as splice enhancer/silencer target sites. This modification holds the potential to govern the process of pre-mRNA splicing**.** (Toms et al., 2020). Lentz et al. (2013) and Wang et al. (2020) used these molecules to restore hearing and vestibular function in USH1C-deficient rodents *(Lentz et al., 2013; Wang et al., 2020).*

The strategies described above are only effective when photoreceptor cells in the retina are healthy. In advanced stages, cell replacement therapies and retinal prostheses may be the only viable treatment options *(Terrell et al.,2019; Nadal et al.,2018).*

1.12. MYO7A- Myosin VIIA

MYO7A gene is one of the most involved genes in the case of Usher syndrome *(Ahmed et al.,2003).* It is located on chromosome number 11q and contains 48 coding exons producing a protein of 2215 amino acids weighing 254KDa (*Weil et al.,1998*). It is broadly expressed in inner ear hair cells, the human kidney, the retina, and the liver *(Adato et al.,2005).* It belongs to the motor genes' superfamily that encodes myosin *(Adalto et al.,1997).* Myosin is of various kinds and performs a wide range of functions in different parts of the body, among which vision and hearing are critical; where it interacts with actin filaments facilitating specialized functions (*Hassan et al., 1995)*. Myosin contains regions designed to engage with other proteins that possess structural attributes enabling the facilitation of ciliary motion within actin filaments (Levy et al., 1997). Through targeted interactions with macromolecules responsiblefor ferrying diverse cargo, myosin effectively propels itself along actin filaments *(Jaijo et al.,2007).* A portion of the myosin has been identified to play a role in the occurrence of hearing impairment. when mutated as in the case of (*MYO6, MYO7A, or MYO15).* However, among these genes, only *MYO7A* is found to be involved in USH, particularly USH1B, which accounts for 70% of USH1 cases *(Jaijo et al.,2007).* Within cochlear hair cells, vestibular hair cells, and photoreceptors (specifically rodsand cones), it is hypothesized that usher proteins *USH1C USH1G*, *CDH23,* and *MYO7A* constitute usher protein network that mediates mechano-transduction (*Alessandro et al., 2022*).Unconventional myosin is encoded by the *MYO7A* gene (*Tengyang et al., 2018; Ahmed et al.,2017).*

Mutations in the *MYO7A* gene, interestingly, can cause non-syndromic dominant or recessive hearing loss as well as Usher syndrome (*Koenekoop et al.,1999*). USH1B is the mostcommon subtype of USH1 and is caused by a mutation in the *MYO7A* gene (*Jaijo et al.,2007*).Severe to profound hearing loss is caused by the mutation of the *MYO7A* gene among usher patients, along with equilibrium disorder and RP *(Jaijo et al.,2007).* However, exceptions are reported, where congenital profound hearing loss with absence of vestibular function occurs due to pathological variants, with early onset of RP with a rapidly progressive nature (*Galbis et al.,2021*).

Figure 1.4 shows a schematic structure of protein *MYO7A* that consists of a motor head domain, followed by a neck region composed of five IQ (isoleucine-glutamine) motifs, and a tail that is made of a coiled-coil domain, two FERM domains, two MyTH4 domains and a Src homology 3 (SH3) domain *(Yen et al., 2010).*

Table 1.3 shows the reported mutation in the *MYO7A* gene causing Usher syndrome in Pakistan (*Riazuddin et al.,2008).*

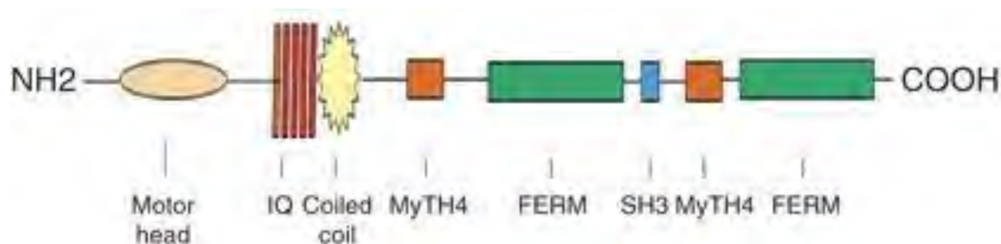

Fig 1.5. Schematic diagram of protein MYO7A showing different domains

Table 1.3. Reported *MYO7A* gene mutation in the population of Pakistan

Sr. No.	Mutation	Predicted Protein Effect	Domain Affected
1.	c.252C>G	p.N84k	Motor
2.	c.398_399insC	p.H133fX139	Motor
3.	c.471_1G>A	Frameshift	Motor
4.	c.495delG	p.E166faX170	Motor
5.	c.495delG	p.E166faX170	Motor
6.	c.495delG	p.E166faX170	Motor
7.	c.460G>A	p.G214R	Motor
8.	c.460G>A	p.G214R	Motor
9.	c.977T>A	p.L326Q	Motor
10.	c.1309G>A	p.D437N	Motor
11.	c.1309G>A	p.D437N	Motor
12.	c.1591C>T	p.531X	Motor
13.	c.1935+1G>A	Frameshift	Motor
14.	c.2476G>A	p.A826T	IQ
15.	c.2914C>T	p.R972X	Posts-c
16.	c.3135_3136insC	p.11046fsX1954	MyTH41
17.	c.3508G>A	p.E1170K	MyTH41
18.	c.3508C>A	p.E1170K	MyTH41
19.	c.3631delIT	p.Y1211fsX1231	MyTH41
20.	c.4838delA	p.E1613fsX1644	SH3
21.	C5146_5148delGAG	pE1716del	D
22.	c.5366+1G>A	Frameshift	MyTH42
23.	c.5944G>A	p.G1982R	FERM2
24.	c.5944G>A	p.G1982R	FERM2

CHAPTER 2

MATERIALS AND METHODOLOGY

2. MATERIALS AND METHODS

2.1. Ethical Approval

The present study was approved by the Bio-Ethical Committee (BEC), Department of Zoology, Faculty of Biological Sciences. Quaid-i-Azam University Islamabad, Pakistan. Written approval from Layton Rahnatullah Benevolent Trust(LRBT) hospital and verbal permission from the Head of Al Ibrahim Trust Eye Hospital Karachi, Sindh, Pakistan, was taken for blood sample collection.

2.2. Identification of Families

Based on clinical investigation, families affected with Retinitis Pigmentosa (RP), syndromic, and non-syndromic families were identified. The patients and their first-degree relatives enrolled in this study were mainly from different areas of Sindh, Pakistan.

2.3. Family Pedigree

After detailed interviews with the normal elders of each family, genetic relationships and details of families were obtained. From pedigree analysis, the mode of inheritance was determined. To draw the pedigree of each family, HaploPainter 1.043 was used. The drawn pedigrees showed different generations represented with Roman numbers. The square in the pedigree represents a male and the circle a female. Hollow symbols show normal individuals, while the filled ones are affected by RP (syndromic/non-syndromic). Deceased individuals have diagonal lines on their symbols. The consanguinity between two individuals, if present, is shown by double lines.

2.4. Exclusion Criteria

- Non-familial cases of retinitis pigmentosa were excluded.
- Patients with other ocular diseases other than RP were also excluded.

- Despite being suitable, patients unwilling to participate in research were not forced and hence excluded.

2.5. Inclusion Criteria

- All clinically diagnosed patients with syndromic and non-syndromic RP with a positive family history of having at least two affected individuals, with all their consent of willingness to participate in this study were included.

2.6. Blood Sample Collection

With the complete consent of families, blood samples of affected and normal individuals (as control) were collected at Al Ibrahim Trust Eye Hospital, Malir, and LRBT Hospital, Korangi, Karachi. 5ml of blood was extracted from the median cubital vein with the help of a syringe and was collected in EDTA vacutainer tubes, having a Unique Anonymous Identification (UAI) number. The name of every individual with a family ID was written on each tube to keep it distinguished. To prevent clotting, blood collected in the EDTA tube was immediately mixed by gentle shaking, which was stored in a refrigerator at -4°C. The affected patients with syndromic/non-syndromic RP were clinically diagnosed and confirmed by the doctors present there.

2.7. Extraction of Genomic DNA

For the extraction of genomic DNA, the following two methods were used.

1: Phenol-Chloroform method.

2: WizPrep gDNA Kit method.

2.7.1. Phenol-Chloroform Method: It is an organic extraction method of isolating genomic DNA, commonly used in molecular genetics. It isolates high-quality and high-yield DNA from any biological sample. The 3-day protocol is given ahead.

2.7.1.A. 1st day of DNA extraction

- EDTA tubes filled with blood samples were kept out of the refrigerator for 10-15 minutes before starting genomic DNA extraction.
- For each sample, an empty Eppendorf tube was labeled with the name and family ID and placed in a wreck in front of the sample to avoid confusion.
- 750 µl blood from each sample was transferred to a similar labeled Eppendorf tube.
- 750 µl of sol A was added in blood, and tubes were left at room temperature for 20-25 minutes.
- Eppendorf tubes were then centrifuged at 13000 rpm for 15 minutes.
- Half of the amount after centrifugation was discarded.
- Sol A (400 µl) was gain added, and centrifugation was repeated.
- Each time, before centrifugation, the pallet was ensured to be broken by vortex or tapping.
- The washing of the blood sample with sol A, as mentioned above, was done three to four times until the pallet appeared clear.
- Once the clear pallet was obtained, the following chemicals were added.
  - ❖ Solution B (400 µl)
  - ❖ Sodium Dodecyl Sulphate SDS (25µl)
  - ❖ Proteinase Kinase PK (5 µl)
- All tubes were vortexed.
- The 1st day of the protocol ended by incubating tubes overnight at 37 °C.

2.7.1.B. 2nd day of DNA extraction

- Overnight left incubated tubes were taken out and kept at room temperature for 2-3 minutes.
- Solution D and C+D were made fresh on the same day (day 2) before use.
- 500 µl of Sol (C+D) was added to each Eppendorf tube.
- After adding the solution, tubes were centrifuged at 13000 rpm for 15 minutes.

- New Eppendorf tubes were labeled to transfer the upper layer from centrifuged tubes neatly.
- 500 µl of Solution D was added in newly labeled Eppendorf tubes filled with a neat layer.
- The tubes are centrifuged again at 13000 rpm for 15 minutes.
- Again, the new Eppendorf was labeled to transfer the newly picked layer.
- The following chemicals are added to each tube.
  - ❖ Isopropyl Alcohol (500 µl)
  - ❖ Sodium Acetyl (60 µl)
- After adding the above-mentioned chemicals, tubes were centrifuged again at 13000 rpm for 15 minutes.
- The whole content from all tubes was discarded gently and carefully.
- 200 µl of 70% ethanol was added, and tubes were centrifuged.
- All tubes were left for drying until no bubbles were left seen (this took 2-3 hours)
- In the last step of day two, 200 µl TE buffer was added to dried tubes and left for overnight incubation at 37 °C.

2.7.1.C. 3rd day of DNA extraction

- Heat shock was given to extracted DNA in a water bath at 70°C for 1 hour. It inactivatesthe nucleases and prevents DNA denaturation.
- After the heat shock, tubes were placed at room temperature for 5 minutes.
- To mix the DNA and buffer properly, a short spin was given to all the tubes bycentrifuging at 3000 rpm for 2 minutes.
- DNA samples were then stored at 20°C in a properly labeled cryo box.

2.7.2. WizPrep gDNA Kit Method: It is a more convenient, easy method to extract genomic DNA within 20 minutes to 2-3 hours depending on the quantity of sample. The processing requires a rapid spin-column format. The protocol is given below.

- Take 200µl of whole blood, add 200 µl of GB Buffer and 20 µl of Proteinase K in an Eppendorf tube.
- Vortex the above mixture and incubate at 56°C for 20 minutes.
- Add 200µl of 100% Ethanol to the above mixture.
- Vortex the tubes to properly mix.
- Transfer the lysate (above mixture) into a column attached to a collecting tube.
- Give a short spin to the column (1 minute at 13000 rpm).
- Discard the filtrate and transfer the column to new collection tubes.
- Add 500µl of W1 Buffer in column and centrifuge for 1 minute at 13000 rpm.
- Discard the filtrate, add 500µl of W2 Buffer, and centrifuge it for 1 minute at 13000 rpm.
- Discard filtrate and shift the column to newly labeled Eppendorf tubes.
- Add 50-100 µl Elution Buffer and keep it at room temperature for 1 minute.
- Centrifuge it for 1 minute at 13000 rpm and discard the column.
- The eluted purified DNA is extracted.
- Store the extracted DNA at 20°C for a few days and at -70°C for long-term storage.

2.8. Agarose Gel Electrophoresis (1%)

Gel electrophoresis was done after DNA extraction for its confirmation. The protocol is given below.

- To prepare 1% agarose gel, 0.5 gm of agarose powder was added in 50ml of 1X TBE (Tris-Boric Acid EDTA) buffer in a conical flask.
- 1X TBE buffer is prepared from 10X TBE buffer by adding 900 ml distilled water in 100ml of 10X TBE in a 1000ml bottle. The 10X TBE buffer is made by adding 0.5M EDTA (40ml), 108 gm Tris, 54gm Boric acid and d, and distilled water, raising the final volume to 1000ml and adjusting pH at 8.
- To make a clear solution, a conical flask (covered with aluminum foil) was kept in the microwave for 2-3 minutes.
- The flask was kept at room temperature for 3-5 minutes to cool down.

- 300 µl of Ethidium Bromide was added to the flask. This is an intercalating agent used for the identification of DNA under UV light (it is overseen carefully, as the chemical is carcinogenic).
- The casting tray and combs were set in a Gel mold.
- Clear solution freshly prepared was poured into the casting tray gently so that no bubbles formed in the tray.
- The poured solution was left at room temperature for 30-40 minutes to solidify (polymerization).
- Before placing the gel in the Gel tank (Clever Scientific Limited, CS-3000V), it was filled by running buffer that is, 1XTBE buffer.
- After the solidification of the gel, the combs were gently removed, and the gel was carefully placed in a gel tank.
- Before loading each sample in the well, 3µl of extracted DNA was mixed with 3 microliters of 6X Bromophenol blue (loading dye) dotted on a parafilm.
- The Gel electrophoresis apparatus was closed and ran at 120 volts for 25 minutes.
- After running, the Gel was carefully visualized under UV light using the Gel Documentation System.

Table 2.1 highlights the chemical concentration and composition used in DNA extraction through the phenol-chloroform method.

2.9. Nanodrop

Using T.E. Buffer as a blank, DNA samples were quantified and confirmed to determine the purity and concentration of isolated genomic DNA using nanodrop (Thermo-Scientific 2000).

Table 2.1. Chemical concentration and composition used in DNA extraction.

Sr No.	Solutions	Concentration and chemical Composition
1.	Solution A	Distilled water 880 ml Sucrose 109.44g (0.32M) Tris 10ml (5mM) pH 7.5 $MgCl_2$ 2.38ml (5mM) 1%(v/v) Trition-100 (10ml)
2.	Solution B	Distilled water 1588 ml Tris 10ml (10mM) pH 7.5 EDTA 2ml (mM) pH 8.0 NaCl 400ml (mM)
3.	Solution C	Phenol (pH 8.0)
4.	Solution D	Chloroform 24 volume, Isoamyl alcohol 1 volume
5.	TE Buffer	10mM Tris Hcl (PH=8.0), 0.1 mM EDTA
6.	SDS	20%
7.	Proteinase Kinase	10mg/ml
8.	Ethanol	70%

9.	Chloroform: Isoamyl Alcohol	24:1
10.	Isopropanol (chilled)	100%
11.	Ethanol	70%

Table 2.2. Composition of Agarose gel and other required chemicals

Sr. No	Solutions	Composition
1.	1% Agarose Gel (50ml)	1X TBE (50ml) Agarose (0.5g) Ethidium Bromide (2µl)
2.	2% Agarose Gel (50ml)	10X TBE (5ml) Agarose (1.0 g) Ethidium Bromide (5µl) Distilled water (45ml)
3.	Gel Preparation buffer (10X TBE)	Boric Acid (0.025M, 27.5g) EDTA (3.6 g, pH 8.3) Tris Base (0.89M, 54g) Deionized water (500 ml)

4.	Gel Running Buffer (1X TBE)	10X TBE (1 part; 10ml) Distilled water (9 part; 90ml)
5.	Ethidium Bromide (50 ml)	Autoclaved filter water (50ml) Ethidium Bromide (0.5g)
6.	Bromophenol Loading Dye (25 ml)	Autoclaved filter water (25ml) Bromophenol blue (0.087 %) Sucrose (10g)

2.10. Primer Designing

Primers were designed using Primer-3 software https://primer3.ut.ee/ to amplify the *MYO7A* gene to analyze mutations and polymorphisms. The general conditions including annealing temperature, size of the amplitude, the concentration of salt, and length of primer were selected for optimum level. The Ensemble website: http://asia.ensemblr.org/Homo_sapiens/Info/Index provided the reference sequence that was essential for building primers. BLAT (Blast Like Alignment Tool) on the UCSC genome browser and the In-Silico PCR tool were used to evaluate the specificity of the chosen primers. Table 2.3 provides additional information on the mutation locus, primer sequence, melting temperature (Tm), and size of the product for primers. Table 2.3 highlights the details of the primer used for the *MYO7A* gene.

Table 2.3. Primers for selected mutation in *MYO7A* Gene

Gene	Exon	Locus	Primer	Sequence (5’….. >3’)	Primer length (bp)	Product Size (bp)
MYO7A	6/7	11q13.5	Forward primer	GAGGGTCCGTATTGTCAGCT	20	556
MYO7A	6/7	11q13.5	Reverse primer	AGCAATACGGGCAGCAATAC	20	556

2.11. Primer Dilution

The ordered primer had a commercial concentration of 50 picomoles/µl and was diluted to a final concentration of 10 picomoles/µl.

2.12. Polymerase Chain Reaction (PCR)

From each of the 4 USH families, the genomic DNA of the proband was amplified using a polymerase chain reaction. The procedure was performed in PCR tubes (Axygen USA) having a capacity of 200µl. The chemicals (Thermo Scientific PCR Kit) and the volume used in the reaction m mixture are given below. Table.2.4. highlights the chemical concentration and volume used for PCR and Table 2.5. highlights its thermo-cycler profile.

Table 2.4. Chemical concentration and volume used in the reaction mixture.

Sr. No	Chemicals	Concentration	Volume
1.	Taq buffer [(NH4)2So4]	10X	2.5µl
2.	dNTPs	2.5mMthe	2.5µl
3.	MgCl2	2.5mM	2 µl
4.	Forward Primer	10pmol/ µl	0.5µl
5.	Reverse Primer	10pmol/ µl	0.5µl
6.	DNA	>100ng/ µl	2 µl
7.	Taq Polymerase	5 U/ µl	0.5µl
8.	PCR Water		14.5µl
9.	Total Volume		25 µl

Before placing the PCR tubes in a thermos cycle (Bio-Rad T100) for the reaction to occur, all tubes are subjected to a short spin in a centrifuge for 2 minutes at 3000 rpm. The thermal conditions set for the PCR reaction are given in Table 2.5.

Table 2.5. Themo cycler profile for Touch Down PCR at 65°C and 55°C

Sr. No	Step	Temperature	Time	Cycle
1.	Initial Denaturation	96°C	5 min	1X
2.	Denaturation	95°C	45 sec	40X
3.	Annealing	55°C	45 sec	40X
4.	Extension	72°C	60 sec	40X
5.	Final Extension	72°C	10 min	1X
6.	Hold	25°C	∞	

2.13. PCR product confirmation

For the confirmation of the PCR product, 2% Agarose gel was prepared by dissolving 1g of agarose in 50 ml of 1X TBE buffer and 2µl of EtBr. Gel Electrophoresis was done by loading (3-4 µl) of the PCR product with 3 µl of loading dye and running at 120 V for 25 minutes. Then the gel is carefully visualized under UV light by using the Gel Documentation System (Cleaver Scientific Limited).

2.14. Purification of PCR product

A purification kit (Thermo-Scientific) was used for the purification of PCR product. The protocol is given below.

- Binding buffer of an equal volume was added to the PCR product.
- The mixture was left at room temperature for 1 minute (after tapping the tubes).

- For proper mixing, tubes were centrifuged at 13000 rpm for 2 minutes.
- Samples were transferred to pre-labeled spin column tubes with attached collection tubes.
- Spin column tubes were centrifuged at 13000 rpm for 1 minute.
- In each sample, 350µl washing buffer was added.
- Tubes were centrifuged at 13000 rpm for 1 minute.
- All samples were kept at room temperature for 2 minutes.
- In each sample, an elution buffer was added (that was kept in an incubator at 70°C before use).
- The spin column tubes were placed in Eppendorf tubes that were pre-labelled.
- The samples were kept at room temperature for 2 minutes.
- The tubes were centrifuged at 13000 rpm for 1 minute.
- To check the purity of DNA, samples were run on 2% Agarose Gel.

2.15. Sequencing

All the samples were sent for commercial Sanger sequencing (Macrogen, South Korea). The purified PCR product (8 µl) was mixed with (4µl) of forward primer, and properly sealed before sending for sequencing. The sequence reactions were conducted using big dye terminator chemistry. The capillary electrophoresis method was used to separate the labeled DNA fragments, and a spectrum analyzer was used to confirm their detection. The spectrum analyzer was used for the later detection. For documentation, each nucleotide base (A, G, T, and C) was labeled with a specific dye. For visualization of Sanger sequencing results, Sequencher 5.6.4 and Chromas and Chromas 2.6.6 were employed.

2.16. Mutational Analysis

Ensemble genome browser http://www.ensembl.org/index.html was used to get reference sequence. The sequence to be analyzed was compared against the reference sequence. Sequencher 5.6.4. The detected mutation was confirmed through Mutation Tester. For further confirmations, other computational tools like PROVEAN https://biotools/provean, and I-

Mutant https://gpcr2.biocomp.unibo.it/cgi/predictorsI-Mutant3.0/1-Mutant3.0cgi were used. Effect on protein level was detected through Uniport https://www.uniprot.org/. Chromas 2.66, SIFT https://sift.bii.a-star.edu.sg/, and ITASER https://zhanggroup.org/I-TaASSER/ were used for the analysis and confirmation of the chromatogram. The effect on protein's structure and chemical nature was determined through the HOPE (Have Your Protein Explained) tool https://www3.cmbi.umcn.nlo/hope/.

CHAPTER 3
RESULTS

3. Results

Twenty-six families, from different regions of Sindh, Pakistan, diagnosed with RP, were included in this research study in a period of 4 months (September 2022-December2022) from Al-Ibrahim Trust Eye Hospital and Layton Rahmatullah Benevolent Trust (LRBT) hospital Karachi. All the families had a positive family history of RP, with consanguineous marriages observed commonly. Among 26 families, 5 were syndromic (Usher syndrome) cases and the rest were non-syndromic. The total number of members from which the blood sample was collected was 121 among which the affected ones were 65 and 56 were phenotypically normal.

Initially, the families were enrolled based on common RP symptoms that are night blindness and visual impairment. Furthermore, the ophthalmologist confirmed the diagnosis based on a fundus examination of affected individuals. Whereas, in syndromic cases, the affected ones had hearing impairment (from very low sensation to complete deafness), with and without equilibrium problems, along with the presence of RP.

Due to the financial limitations of funding, probands from 4 Usher syndrome families (RP-69 aged 24, RP-73 aged 39, RP-83 aged 18, and RP-150 aged 28 years) were selected for mutational analysis of exons 6/7 of the *MYO7A* gene. The clinical examination of probands from each of these 4 selected families showed congenital deafness with various onset of retinitis pigmentosa and the presence of vestibular dysfunction in 2 to 4 individuals.

Figure 3.1 shows the fundus photographs of a USH-affected individual from one of 4 selected families (RP-69) with prominent bony spicules and attenuated vessels, pigmentation, and cataract in the left eye; clearly showing symptoms of RP.

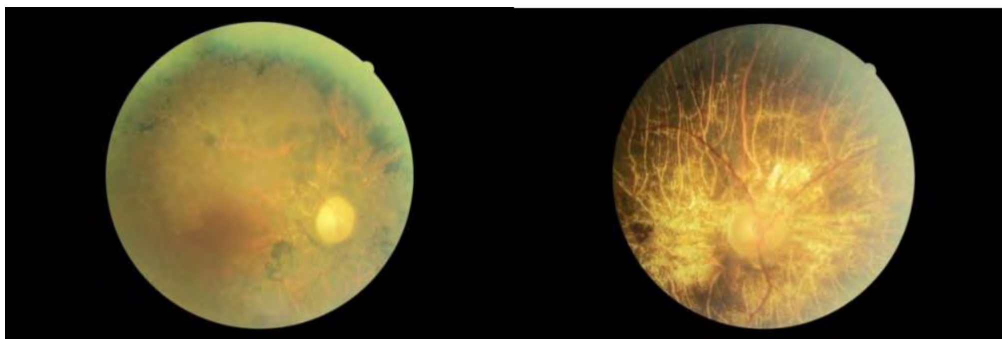

Figure 3.1 Fundus examination of a patient with Usher syndrome (RP-69-V.III)

3.1. Detailed demographic, clinical, and family history of each of the four selected USH-affected families

3.1.1. Family RP-69 (Usher family 1)

The family RP-69 belongs to Karachi, Sindh, Pakistan. There are 5 Usher affected individuals in this family, with one deceased and 4 alive. The deceased individual was congenitally deaf and had complications in night vision; died due to the onset of high fever by the age of 4 years. To genetically analyze, a blood sample from the proband (V.III) and her USH-affected sister was taken. Blood samples of the other 2 unaffected siblings and mother were collected as control. Descriptive data was collected from the elders of the family. All the affected ones were congenitally deaf, with bilateral RP and ataxia. Figure 3.2 shows the pedigree of a family with an autosomal recessive pattern of inheritance.

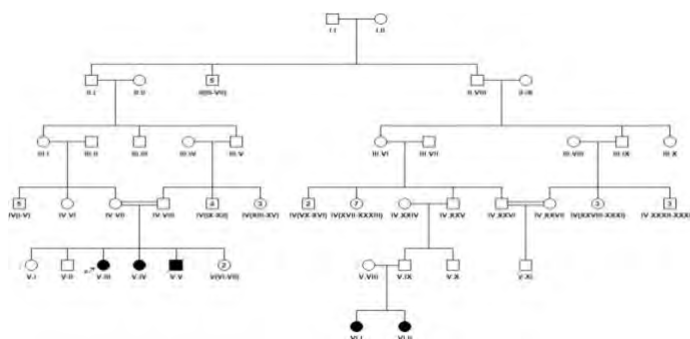

Figure 3.2 Pedigree of RP-69 (USH family 1)

3.1.2. Family RP- 83 (Usher family 2)

The family RP-83 belongs to Karachi, Sindh, Pakistan. There are 5 affected individuals in the family. The proband (III.I) and one of his siblings (III.III) were affected by USH. Both were congenitally deaf and had complications in night vision with normal vestibular function. Other affected members II(II.VII), III (III.XXIV), and III (XX.IX) had bilateral RP only. However, due to the unavailability of individuals with non-syndromic RP, the blood samples of 2 usher-affected individuals and their father were collected. Descriptive data was collected from the elders of the family. Figure 3.3 shows the pedigree of this family with an autosomal recessive pattern of inheritance.

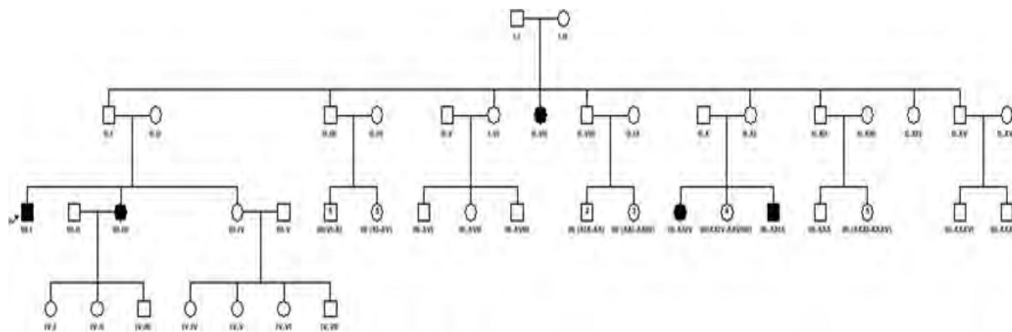

Figure 3.3 Pedigree of RP-83 (USH family 2)

3.1.3. Family RP-73 (Usher family 3)

The family RP-73 belongs to interior Sindh, Pakistan. There are 13 RP-affected individuals in this family, with 2 syndromic and 11 non-syndromic. The proband (III.I) and her sister (III.III) were affected by Usher syndrome. They were congenitally deaf, with impairment in night vision and normal vestibular function. While the other members had progressive RP only, with symptoms like night blindness, photophobia, and poor peripheral vision. Blood samples from all 13 affected and 12 unaffected individuals were taken for genetic analysis. Descriptive data was collected from the elders of the family. Figure 3.4 shows the pedigree of family RP-73 with autosomal recessive inheritance.

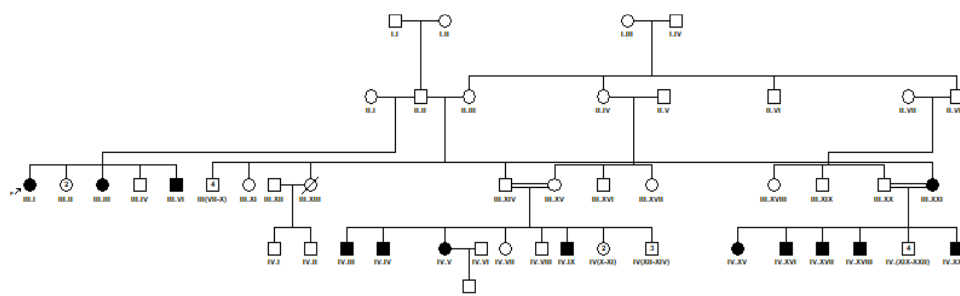

Figure 3.4 Pedigree of RP-73 (USH family 3)

3.2.4. Family RP- 150 (Usher family 4)

The family RP-150 belongs to Peshawar, KPK, Pakistan. There are 3 USH-affected individuals in this family. The proband (IV.I) had Usher syndrome, with progressive RP, congenital hearing loss, and normal vestibular function. His sibling (IV.V) was completely blind by the age of 15 years with congenital hearing loss and normal vestibular function. Whereas the 3rd affected individual (IV.VI) had progressive RP with low hearing sensation and normal vestibular function. Descriptive data was collected from the elders of the family. Figure 3.5 shows the family pedigree of family RP-150 with autosomal recessive inheritance.

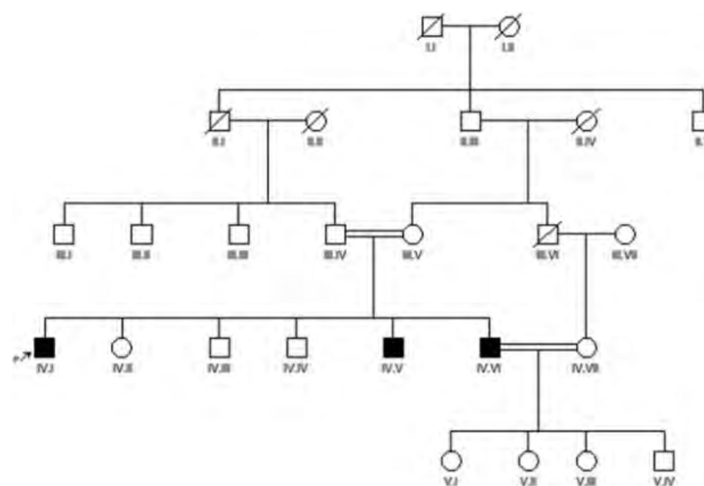

Figure 3.5 Pedigree of RP-150 (USH family 04)

3.2. DNA Isolation

From each blood sample, DNA was extracted from the white blood cells. The mean concentration of extracted DNA was up to 50 ng/µl with a purity level of 1.8. Spectrophotometry was done to record the DNA content that is given in Table 3.1. To check the integrity of genomic DNA, 3 µl sample was run on Agarose gel. Figure 3.6 shows the results of isolated DNA from blood samples of USH-affected families.

Figure 3.6 Agarose Gel electrophoresis results showing DNA bands for 13 individuals used for molecular analysis.

Table 3.1 Nanodrop results of the probands of USH families.

Family ID	Pedigree ID	Concentration (ng/ µl)	A 260/280
RP-69	V.III	196 (ng/ µl)	1.79
RP-73	III.I	185 (ng/ µl)	1.76
RP-83	III.I	241 (ng/ µl)	1.74
RP-150	IV.I	163 (ng/ µl)	1.76

3.1.Primer Optimization

Gradient PCR was used for the optimization of primer pairs. For mutational analysis of exon 6 and 7 of the *MYO7A* gene in Usher patients, the annealing temperature of 65°C was found suitable with good amplification of PCR products.

3.1. Polymerase Chain Reaction

To amplify the DNA samples, a Polymerase Chain Reaction (PCR) was performed. Figure 3.7 shows the amplified DNA bands run on Agarose gel (2%). Table 3.2 shows the ingredients and their chemical concentration used.

Figure3.7. PCR results of amplified DNA

Table 3.2. Chemical concentration and volume used in the reaction mixture.

Sr.No	Chemicals	Concentration	Volume
1.	Taq buffer [(NH4)2So4]	10X	2.5µl
2.	dNTPs	2.5mM	2.5µl
3.	$MgCl_2$	2.5mM	2 µl
4.	Forward Primer	10pmol/ µl	0.5µl
5.	Reverse Primer	10pmol/ µl	0.5µl
6.	DNA	>100ng/ µl	2 µl
7.	Taq Polymerase	5 U/ µl	0.5µl
8.	PCR Water		14.5µl
9.	Total Volume		25 µl

3.2. PCR product purification

A purification kit (Thermos Scientific) was used to purify PCR products. All the PCR products of probands were purified and eluted in an elution buffer. The confirmation of amplicon was obtained by loading 2% agarose gel electrophoresis with 4 µl purified PCR product.

3.3. Sanger's Sequencing

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

3.3. Genetic Analysis

Mutations in the *MYO7A* gene are known to cause usher syndrome in various populations globally. The hotspot exons 7 of *MYO7A* gene (MIM: 276903) having a base pair length of 556 was selected to screen mutations and polymorphism in 4 probands of selected usher families. The reference sequence of exon 7 of the *MYO7A* gene was obtained from Ensemble and was used as a standard sequence against the sequence of selected USH probands that were to be analyzed.

Among the selected 4 families of USH, Sanger sequencing of proband RP-69 showed disease-causing variants in exon 7 of the *MYO7A* gene. However, no sequence variant was detected in the other 3 families in the analyzed exon, which highlights the need to analyze other exons for the detection of mutation or polymorphism.

A reported disease-causing variant (c.640G>A) was detected through Sanger's sequencing in exon 7 of the *MYO7A* gene in the proband of RP-69. This variant causes a change in the sequence of amino acids in protein structure, causing changes in the splice site;

affecting proteinstructure and halting it from performing its function. It severely impairs the ATP binding function of the protein, causing the onset of USH-IB *(Adalto et al.,1999).* The affected ones are congenit ally deaf, with various onset and progression of RP and vestibular dysfunction, showing all the symptoms of USH1B that could be caused by the disease-causing variant found. Figure 3.7 shows the analyzed results from Sequencher 5.6.4 where a nucleotide G is replacedby A.

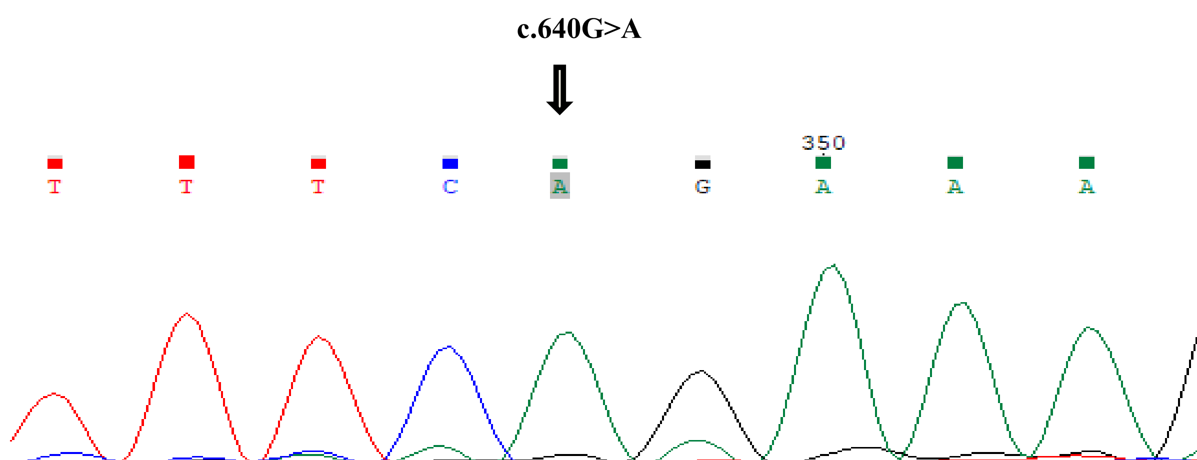

Fig.3.7. Sequencing electropherogram showing single nucleotide change at position c.640G>A in exon 7 of *MYO7A* gene of proband (V.III) RP-69.

Table 3.2 Summary of sequence variation identified in exon 7 of *MYO7A* gene taken in this study.

Family ID	Chromosomal location	Effect	rs.ID	Mutation Tester	Amino acid change	Status
RP-69	Chr11.78867955G>A	Splice site changes: protein features affected.	rs.43327	c.640G>A	G214R score:125	Reported

CHAPTER 4

DISCUSSION

4. Discussion

Usher syndrome is the most prevalent cause of deaf-blindness worldwide, with a prevalence of 4 to 17 per 100,000 people; causing simultaneous loss of hearing and vision with vestibular dysfunction in some, but not all cases (*Toms et al.,2022)*. It has an autosomal recessive inheritance pattern. (*Koenekoop et al.,1999).* Three primary clinical types of USH are 1, 2, and 3, each of which is further subdivided into 14 subtypes, each caused by mutations in a distinct gene up to the present time, it has been observed that disease-associated mutations within nine specific genes are responsible for triggering Usher syndrome (USH). These genes encompass *MYO7A*, *USH1C*, *CDH23, PCDH15, and USH1G* (also known as SANS), linked to Usher type 1. Similarly, *USH2A, ADGRV1,* and W*HRN* contribute to Usher type 2, while *CLRN1* is associated with Usher type 3 *(Alisandro et al.,2022).* The regions of the retina with prominent expression of usher proteins include the connecting cilium, periciliary membrane, calyceal process, inner and outer segments, basal body, and synapse, where any mutation could alter protein structure and ultimately lead to progressive deterioration and functional loss of structures. Particularly, the rods are affected first, which makes it difficult to observe in the dark. A "tunnel view" is often the first symptom of cone degeneration, and continued degeneration results in loss of central vision (*Mathur et al.,2015*). USH has great genetic variability; some of the genes are also present in non-syndromic RP andnon-syndromic hearing variants. In people with less awareness and average research practices, the significant variability and rarity of USH sometimes lead to a delayed or inaccurate diagnosis *(Kaenekoop et al., 1999).* Alternative reasons for genetic heterogeneity encompass instances of diverse mutations occurring within the same gene across distinct individuals, as exemplified by the range of MYO7A alleles tied to different phenotypes. Additionally, geneticheterogeneity can arise from alleles impacting multiple genes, or from variations in penetranceand expressivity leading to a spectrum of phenotypes. Interestingly, within the context of Ushersyndrome types 1 and 2, nearly complete penetration rates of around 99-100% have been observed *(Alessandro et al., 2022). MYO7A* mutations, which have been extensively discussed in the literature, cause severe prenatal-to-profound hearing loss, balance issues, and the early start of RP in Usher patients *(Koenekoop et al.,1999).* There are known exceptions, where most pathogenic variations often cause significant congenital hearing loss without vestibular function, in addition, RP has an early start and is severe and fast progressing (*Galbi et al.,2021)*

Large differences in the prevalence of USH have been observed, and the prevalence rateis not fully documented internationally, which is likely because there are insufficient resourcesfor diagnosis and rehabilitation (*Castiglione et al.,20220)*. The global approximation stands at1 in 30,000 individuals, a conservative average considering that alternate investigations have unveiled a wide spectrum of occurrence ranging from 1 to 17 in every 100,000 persons *(Espinos et al.,1998*). There is a lot of variation in the occurrence of various clinical kinds and genetic variants. USH2 is the most prevalent kind (60%) and the USH2A gene accounts for 80–90% of cases. According to a large body of research greater diversity and variability across nations and ethnic groups may be found in USH1. The higher occurrence of USH1 compared to USH2 in specific regions, like northern Scandinavia where USH1B and USH1D are found, and in Louisiana, USA where USH1C is prevalent, can be attributed to the historical presence of these regional isolates *(Castiglione et al.,2022).* Globally, USH3 is uncommon, except for Finland, where its incidence is around 40%, 34% for USH1, and just 12% for USH2 (*Colombo et al., 2021*).

Numerous studies have shown that consanguineous marriages often result in the transmission of autosomal recessive characteristics, and Pakistan is one of the nations where such marriages are quite prevalent *(Ullah et al.,2018).* As, the kids of consanguineous couples are more likely to develop autosomal recessive illnesses, including USH, due to greater homozygosity via descent *(Coellner et al.2018).*

The *MYO7A* gene is predominantly linked to Usher type I. DFNA11 [MIM 601317] (Liu et al., 1997b) and DFNB2 [MIM 600060] (Liu et al., 1997; Weil et al., 1997), as well as atypical forms of Usher syndrome, which are clinically like Usher syndrome type III (*Liu et al., 1998*).

Due to the high prevalence of USH1 in ethnically and variably diverse nations, and the reported knowledge of the *MYO7A* gene being the most common cause of the disease when mutated, we targeted one of its hotspot exons i.e. 6 and 7 of *MYO7A* to analyze its mutation and polymorphism in probands of 4 Usher syndrome families belonging to different ethnic groups of Sindh, Pakistan.

In the given results, a reported disease-causing variant has been found in one of the four probands of selected families screened in this study. However, the probands of the other three

families (RP-073, RP-082, and RP-150) do not show any sequence variation in the targeted exon 7 of *MYO7A* highlighting the need to look for other exons for the detection of causative mutation and polymorphisms.

In family RP-69 (Usher family 1) the proband (V.III) got a clinical diagnosis of congenital hearing loss, progressive RP that developed by the age of 12 years, and vestibular dysfunction. The genetic analysis of exon 7 of *the MYO7A* gene of the proband (V.III of family RP-69) has been found carrying a missense mutation substituting single amino acid at position 214 (p.Gly214Arg); that is a previously reported disease-causing variant, with **rs ID:43327** causing Usher syndrome type 1B, known as the most severe type of USH.

This disease-causing variant was reported first in one of the 13 screened families in a research study reported in 1997, where a Moroccan family with consanguineous parental marriage, showed 2 USH1B affected individuals, carrying (c.460G>A) mutation in exon 7 (*Adalto et al., 1997*).

It has also been reported in the Spanish population in a study of mutational screening of the *MYO7A* gene in 48 unrelated US1B families, where 3 families had been found carrying the same disease-causing variant (c.640G>A) in exon 7 of *MYO7A* among the 24 other mutations (*Jaijo et al.,2006).* Whereas in another study on Spanish people, 183 patients with USH were analyzed against a genotyping microarray in which were detected 98 pathologic alleles corresponding to 32 different mutations, among which the most prevalent gene reported causing USH1B is *MYO7A (Jaijo et al.,2009).*

Whereas, in Pakistan, the (c.460G>A) variant was reported first in the year 2008, where 17 homozygous mutant *MYO7A* alleles were detected in 23 families affected with USH1, including (c.460G>A) (*Riazuddin et al.,2008).* USH1 locus and allelic heterogeneity in Pakistan is comparable to that described for USH1 in the United States and the United Kingdom. *(Hope et al., 1997; Astuto et al., 2000;)* We have found the same disease variant in one of 4 usher families in our study, belonging to the Muhajir ethnicity of Karachi, Pakistan.

In silico analysis of the found variant shows splice sites change. As the consequence of splice, site change is not negligible and holds all the potential of producing aberrant mRNA molecules, which might result in nonfunctional or changed proteins, the mutation is highly pathogenic.

It has been known through research that the mutation (c.460G>A) in exon 7 of *MYO7A* protein occurs in the head domain of the protein and causes severe impairment in the ATP binding function of the protein (*Adalto et al.1999*), which could be a possible reason of causing impairment in the functioning of eye and ear and ultimate development of the disease, usher syndrome, as seen in the proband of the targeted family (RP-69), where the affected ones, is congenitally deaf, with progressive RP and vestibular dysfunction.

4.1. Conclusion and Future Perspectives

In conclusion, all four families used in this study have Usher syndrome with a confirmed clinical diagnosis of RP, hearing loss, and vestibular dysfunction in 2 among 4 families. Due to a lack of budget, just 4 probands, 1 from each family were selected for Sanger's sequencing of exons 6 and 7 to analyze the mutation. Among these, RP-69 was identified to have disease disease-causing variant (c.640G>A) in exon 7, proving the location as one of the hot spots for the Pakistani population having usher syndrome, while the other 3 families require further sequencing. Also, our study aligns with a large body of research which illustrates that USH1B is mostly found in ethnically diverse nations and Pakistan is undoubtedly among those nations. Moreover, genetic counseling was given to all the affected families to avoid transmission of autosomal recessive traits by consanguineous marriages that could lead to the incidence of syndromes like USH. However, the study needs more research innovations and funding, where whole genome sequencing will bring more insight, as the disease is highly heterogeneous.

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