Genetic Basis of Nystagmus in Affected Families

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FINAL APPROVAL

It is certified that we have read the thesis **"Genetic Basis of Nystagmus in Affected Families"** Submitted by Mr. Tahir Aman and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic University, Islamabad for the M.S Degree in Biotechnology.

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for the award of the degree of MS Biotechnology

DEDICATION

This thesis is dedicated to my loving parents. Without their knowledge, wisdom, and guidance, I would not have the goals I have to strive and be the best to reach my dreams!

DECLARATION

I hereby declare that the work present in the following thesis "**Genetic Basis of Nystagmus in Affected Families**" is my own effort, except where otherwise acknowledged and that the thesis is my own composition. No part of the thesis has been previously presented for any other degree.

Date 31-08-2016

(Tahir Aman)

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In the name of ALLAH the most Merciful and Almighty without the help of Whom I was unable to complete my thesis. All respects to Holy prophet MUHAMMAD (S-A-W-W) the most exalted among human being ever born on surface of earth, Who is forever guidance and knowledge for Humanity as whole.

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(Tahir Aman)

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LIST OF ABBREVIATIONS

Abbreviations	Complete Words
SDN	Sensory defect nystagmus
CIN	Congenital idiopathic nystagmus
NN	Neural nystagmus
SSN	See –saw nystagmus
PAN	Period alternating nystagmus
OMIM	Online Mendalian inheritance in man
FISH	Fluorescence in situ hybridization
OD	Optical density
SAP	Shrimp alkaline phosphatase

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ABSTRACT

Nystagmus is an involuntary, rapid and repetitive movement of the eyes that can vary between slow and fast usually involving both eyes. Most commonly the movement is side-to-side (horizontal), however it can also be up and down (vertical) or circular (rotary). Normally, gaze fixation happens with the head movement and stimulation of semicircular canals. Nystagmus occurs when semicircular canals are stimulated without head movement. Nystagmus is caused by gene FRMD7, composed of 12 exons located on X-chromosome. This study was designed to find the pathogenic variants that lead to nystagmus in affected individuals of collected family PKNYS-09. Direct sequencing approach was used after identifying the mode of inheritance in the affected family. Most susceptible exons (9, 10 and 11) were selected for sequencing based on clinical analysis, X-linked mode of inheritance of the pedigree and from previous findings. After sequencing and analysis, no pathogenic variant was observed in the affected individuals of this pedigree. This could be due to the reason that the selected exons were sequenced based on already reported mutations from different world populations and reports on molecular genetics of nystagmus is scarce in Pakistani population. There is a chance of involvement of some other exon whose mutation is leading to nystagmus in the PKNYS-09 pedigree. Also, some mutation may be present in the 5' and 3' untranslated regions (UTRs) of the gene or may be in promoter regions. Further study is required to find the mutated exon of the gene. However, if no pathogenic variant is observed in all the exons and the regulatory regions of the gene, there could be the possibility of involvement of any other gene. Whole exome sequencing can then be performed to find the causative region of the exome. Phylogenetic analysis of FRMD7 nucleotide and peptide sequence reveal highly conserved sequences among different species.

Chapter 1

INTRODUCTION

Nystagmus is a disorder of eye movement which can be defined as a rapid and involuntary movement of eye ball that may be to and fro or may be rotatory (Leigh and Zee, 1999).The word nystagmus is basically derived from a Greek word 'nustagmos' meaning nodding of the head or drowsiness. The word is thought to have evolved due to a comparison with slow downward drift and sudden upward jerk of the head during drowsiness (Oxford University Press, 2006).

There are three main regulation mechanisms in human for maintaining steady gaze including fixation movements, vestibule-ocular reflex and gaze-holding system (Leigh and Zee, 1999). If any one of these regulation system fails, it can originate destruction of steady fixation. Abnormal fixation is of two style, nystagmus and saccadic oscillations. The distinction is only in initial movement. In saccadic oscillations first anomalous movement could be a quick saccadic movement. Within the case of disorder of nystagmus, it's a slow drift or 'slow part away from the target (Leigh and Zee, 1999). It has been suggested that nystagmus results from an anomaly within the gaze-holding pathways or the tissues comprising brainstem mechanism process that originate tonic innervations required to keep up lateral gaze (neural integrator) (Leigh and Zee, 1999, Abel, 1990, Dell, 1990).

Nystagmus is an unconscious eye movement of one or both eyes in any fields or gaze. Eye movements can be anxious, slow, pendular, or rotatry. If rotation of head occur through any point, clear seeable picture are continued through movement of eyes in the opposed side on the relevant point (Medline 2013). They send signal to the nucleus for movement of eye in the brain;

signal is relay to the extra ocular muscles from here, for allowing one's gaze to fixation on single object when the head moves. When the semicircular canals stimulate then nystagmus occur, while the head not in motion. Ocular movement direction is related to semicircular canal which is stimulated (Saladin 2012)

Broadly nystagmus is divided into three categories. Congenital nystagmus, Infantile nystagmus and non-inheritable or acquired which may be due to disorder or drug toxicity. (Leigh and Tannen 1995; Leigh Averbuch 1998). Congenital Nystagmus is mainly by birth and mostly starts at the age of two or three months. Eyes mostly move in horizontal fashion in congenital nystagmus. Additionally it is related to alternative conditions like birth defect, inborn absence of the iris, undersize optical nerves, and also by birth cataract. Infantile nystagmus typically happens between six months to three years of age, and cured mostly by itself between two to eight years of age. Those children which are suffering from infantile nystagmus show symptom of head nodding and tilting. Nystagmus of this kind typically doesn't need treatment. The third type is known as acquired nystagmus which develop in later childhood. The disease causing reason is still unknown. But its main cause is defect in nervous system and certain metabolic disorder such as drug toxicity and alcohol etc.

Nystagmus is caused by gene named as FRMD7. This gene is composed of 12 exons.

The functional region of FRMD7 undergoes mutation and cause nystagmus. Totally 22 Mutation reported in gene FRMD7 (Tarpey et al 2006). Total mutation known in FRMD7 is cosegregated with disease in linked families. X chromosome takes a singular place in the history of medical biological science. Identification of X-Linked disorder is increased by the relative easy knowing this type of inheritance. Moreover, a disproportionately sizable amount of unwellness conditions

are related to the X chromosome as a result of the external appearance consequence of a recessive mutation that is disclosed directly in males. This happens as a result of all males is constitutionally hemizygous for any factor having no active counterpart on the Y chromosome. Thus, though the X chromosome contains solely four percent of all human genes, virtually 10% of disorders with a Mendelian pattern of inheritance are allotted to the X chromosome (307 out of 3,199; info obtained from the OMIM2TM repository (Online Mendalian Inheritance in Man). Present study was designed to study the molecular players of nystagmus in affected individuals of collected family. Direct sequencing approach was used after identifying the mode of inheritance in the affected family. Most susceptible exons were sequenced in the collected family, however no pathogenic variant was observed which indicates that some other exon may be involved in this family. Further study will reveal the causative region for nystagmus.

Chapter 2

LITERATURE REVIEW

Nystagmus is eye disorder in which involuntary movement of eye ball occur. This is basically genetic disorder and transmits due to chromosomes. It may be autosomal or sex chromosomal. It is mainly occurring due to mutation in chromosome and specific gene FRMD7. When mutation occurs in it, then eye ball move involuntarily. Eye ball composed of various parts. Descriptions of eye parts are given.

Cornea:

This tissue layer is the outermost covering of the eye. The cornea is of dome like structure and its function is to protects your eye from those material that would cause injury to the inner elements of the eye. Cornea is composed of several layers, making a protective hard and tough layer that gives further protection. These layers recreate very rapidly, serving to the eye to eliminate injury a lot of simply. This tissue layer suitably permits the eye to properly target light with a lot of effectiveness.

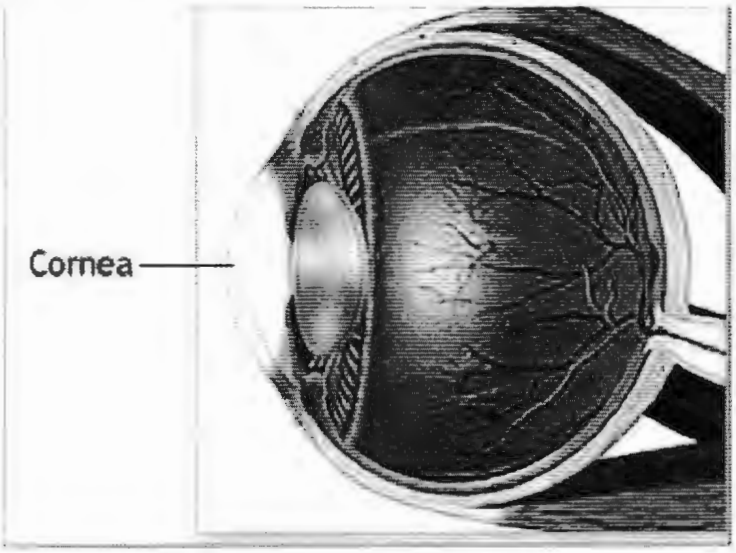

Figure 2.1. Cornea of the eye (adapted from www.youreyedoc.com)

Sclera:

The sclerotic coat is often mentioned as "whites" of the eye. This can be a sleek, white layer on the outer side, however the inner side is brown and composed of grooves that facilitate the tendons of the eye to attach properly. The sclerotic coat provides structure and also helpful in the safety for the inner workings of the eye, however is additionally it is versatile and flexible in order that the eye will move and rotate on every side to sort out out objects as necessary.

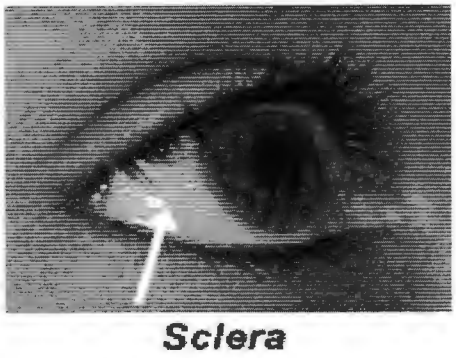

Figure 2.2. Sclera(white) of the eye (adapted fromwww.daviddarling.info)

Pupil:

The pupil seems as a black dot within the middle of the eye. This black region is basically a hole that takes in light , that the eye will focus on the objects ahead of it.

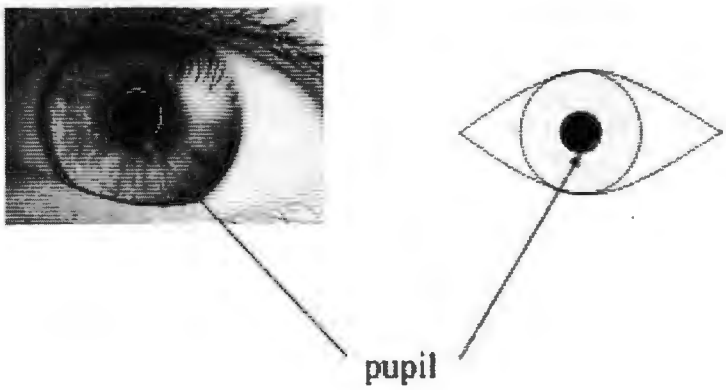

Figure 2.3. pupil as a black dot in the center of eye (adapted from pmgbiology.com)

Iris:

The iris is that space of the eye that contains the pigment which provides color to the eye. This region surrounded the pupil, and uses the dilator pupillae muscles to widen or shut the pupil. This permits the eye to require in additional or less light counting on however bright it's around you. If it's too bright, the iris can shrink the pupil so the eye will focus a lot of effectively.

Figure 2.4. Iris of eye containing pigment (adapted from www.floridaeyespecialistinstitute.com)

Conjunctiva Glands:

These are a unit layers of secretion of mucus that facilitate keep the surface of the eye dampish. If the eye becomes dries then it will become unquiet and painful. It may become a lot of at risk of injury or infection of conjunctiva gland. If these mucous secreting glands become infected, the patient can develop disease known as "pink eye."

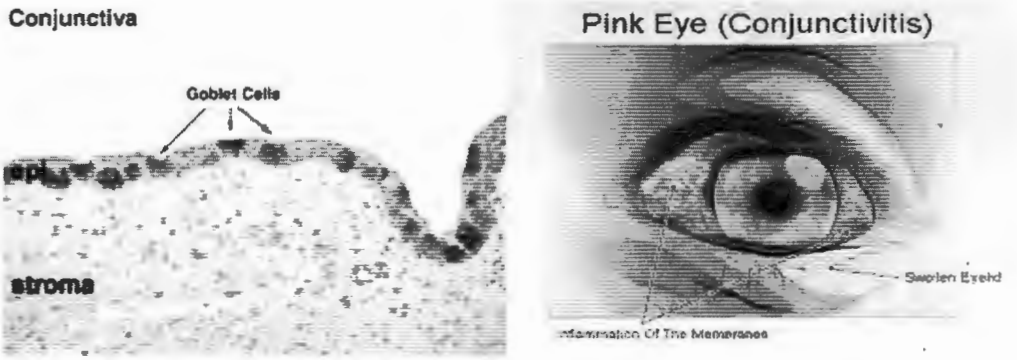

Figure 2.5. conjunctiva gland for eye dampish (adapted from www.oculist.net)

Lacrimal Glands:

These glands are a unit placed on the outer corner of every eye. They manufacture tears that facilitate moisten the eye once it becomes dry, and remove out those particles that cause irritation of eye. A tear removed out probably dangerous irritational material, so proper focusing for eye become easy.

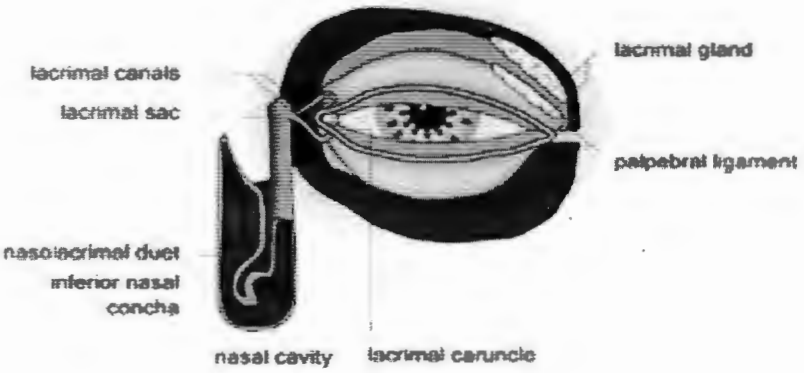

Figure 2.6. lacrimal gland of eye responsible for tear (adapted from www.dryeyezone.com)

Lens:

The lens is present just behind the pupil. This can be a transparent layer that focuses the light rays which the pupil takes in. it's control in situ by the cilliary muscles, which permit the lens to vary its shape looking on the quantity of light rays that hits it thus it is accurately focus.

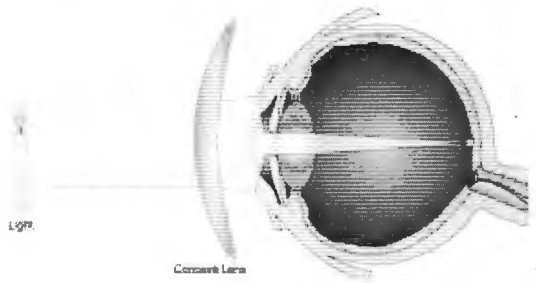

Figure 2.7. lens of eye for focusing of light (adapted from www.passmyexams.co.uk)

Retina:

The light rays focuses by the lens are transferred onto this tissue layer known is retina. This can be composed of rods and cones which are organized in layers, which is able to transmit light into chemical and electrical pulses. This tissue layer is found within the back of the eye. Retina has also connected to optic nerves that may transmit the photographs the eye sees to the brain so that these photographs are completely analyzed here. The back side of this tissue layer, referred to as the macula, can facilitate interpret the minute information of the thing the eye is functioning to interpret. The middle of the macula, referred to as the fovea can enhance the information of those pictures to a perceivable point.

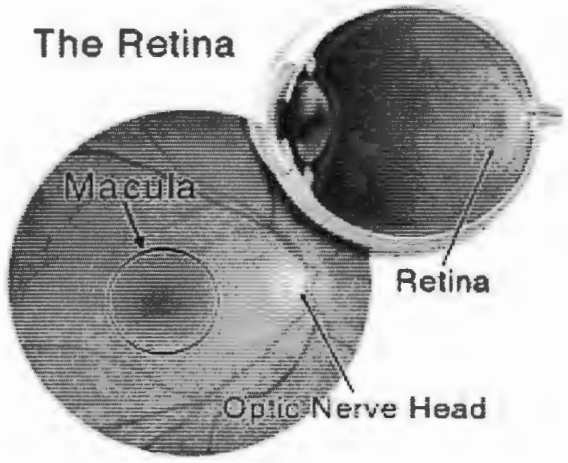

Figure 2.8. Retina for light transmission (adapted from www.floridaeyespecialistinstitute.com)

Ciliary Body:

This tissue layer is of rounded shape that functions in holding and also controlling the movement of the eye lens, and thus, it helps to regulate the specific form of the lens.

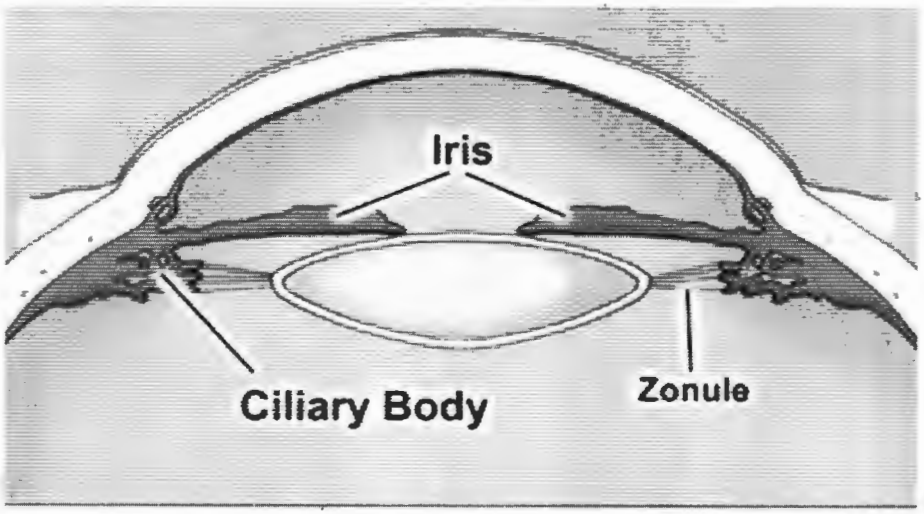

Figure 2.9. ciliary body of eye (adapted from www.keyword-suggestions.com)

Choroid:

This tissue layer lies between other two layers which are retina and sclera that provides blood to the eye.

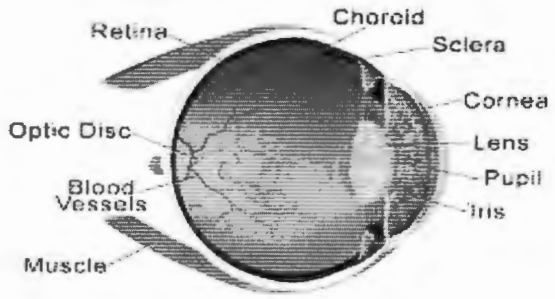

Figure 2.10. Choroid provide blood to eye (adapted from atlasofophthalmology.org)

Humor:

Vitreous humor is a gel which should be set within the back of the eye that helps it hold its form. Vitreous humor takes in nutrients from the tissue layer of cilliary body, aqueous humor and also the retinal vessels therefore the eye will stay healthy. Once junk approaches into the vitreous humor, it causes the eye to realize "floaters," or spots that move across the vision region that can't be attributed to things within the surroundings

Aqueous Humor:

The equoes humor fills the eye and it is watery material. It's divided into two chambers which are exterior and posterior in position. The anterior one is present in iris front, and the next one which is posterior is directly behind it. Both layers permit the eye to sustain its original shape. This liquid is drained through the Schlemm canal in order that any buildup within the eye is removed. If the patient's liquid body substance known is aqueous humor isn't exhausting properly, they'll develop eye disease.

Nystagmus is involuntary condition or may be voluntary in occasional cases. (Zahn 1998) eye movement may developed during at beginning or later in life, lead to reduced or restricted vision attributable to uncontrolled or involuntary movement of the eye ,usually it's known as "dancing eyes"(Weil 2013). Both type of nystagmus which is congenital and infantile are present as a conjugate, eye horizontal vibration and oscillations, eccentric gaze, usually with a most well-liked head flip and tilt. alternative connected options with this might contain gently reduce sharp-sightedness, strabismus, astigmatism, and infrequently head cernuous and nodding movement of eye recordings tell that infantile nystagmus is basically a horizontal jerk wave shape, with a diagnostic fast speed slow stage however pendular and the triangular wave shape may additionally be occur. Nystagmus might occasionally be vertical. as a result of these patients typically have traditional visual modality, it's assume that the nystagmus characterizes a key problem within the elements of the brain accountable for ocular motor control; that the disorder has sometimes been termed 'congenital motor nystagmus (Tarpey et al., 2006; Shiels et al., 2007). Identification of those genes which are concerned with rare conditions yields vital

biological insights .For example, discovery of mutations within the SH2D1A gene (Coffey *et al,* 1998) (involved in sex chromosome mainly of x chromosom lympho proliferative unwellness (XLP, OMIM 308240) which lead to knowing and identification of a brand new intercessor of signal transduction between T and NK cells, and a completely unique family of proteins concerned within the regulation of the immune reaction and response. Discovery of genes for such type and also for different rare, inheritable disorders is of important and valuable in extension of our understanding of basic new processes in human biology, and also the annotated sex chromosome can additional facilitate this process (Ross *et al,* 2005).

These genes situated within a place of the sex chromosome known as the X- inactivation Centre (XIC/Xic). It is believed that the two X chromosomes within a cell physically interact (observed using fluorescence in situ hybridization (FISH)) at which time the XIC from each chromosome are in direct contact. Following this, the TSIX mRNA levels on the chromosome to be inactivated, rapidly plummet for unknown reasons. This reduction in TSIX causes alterations in the chromatin structure of the DNA at the XIC region resulting in a closed, hetero-chromatinized structure.

Paradoxically, this causes the XIST gene in this region to increase expression and XIST mRNA then coats the X chromosome to be inactivated, rendering it inactive. TSIX also interacts with a molecule that attaches methyl groups to the XIST promoter, which inactivates it. This possibly represents a secondary mechanism for ensuring the XIST gene is inactivated on the active X chromosome. The X-inactivation that ensues is believed to be random as to whether the maternal or paternal X is inactivated. Tissue ratio and binomial distribution are follow.

This phenomenon has been used to explain the observation of manifesting female carriers of X-linked recessive diseases due to variable degrees of silencing the X chromosome harbouring the disease gene. However, some tissues have been shown to exhibit skewed X-inactivation such that its constituent cells tend to show inactivation of one or other X chromosomes to a greater degree than would be expected by chance. Again this phenomenon has been used to explain manifesting female carriers of X-linked recessive diseases where females have a phenotype which is equally as severe as affected males suggesting near total silencing of the 'normal' X chromosome.

The process of underlying skew X- One of two X chromosomes is at random inactivated in every cell early in feminine embryonic development (Lyon, 1961) in order that it becomes transcriptionally inactive and thereby equalizes the indefinite quantity of X-linked genes between males and females. Each daughter cell then inactivates the same X chromosome as its parent such that cells are clonally expanded with a particular X chromosome inactivated. Afterward this time, methods able to differentiate between the two X chromosomes is accustomed track the fate of the (Ross *et al,* 2005) totally different cellular populations. At the beginning, differentiation was achieved victimization polymorphic accelerator or enzyme variants (Oni *et al* 1970), but, a lot of recently, the actual fact that placental mammals methylate the inactive sex chromosome and some restriction enzymes by selection cut the unmethylated allele, has allowed a lot of widespread uses (Vogelstein *et al,* 1985).

Above all, the human steroid or androgen receptor locus has been found to be helpful because of its enhance degree of hetrozygosity (about 90%) (Allen *et al,* 1992). curiously, several tumors, as

well as leukaemias, myelodysplasia, and myeloproliferative disorders, are shown during this way to show and express just one sex chromosome which are x, implying derivation from one cell (Fialkow, 1976, Anger, *et al*, 1990). This process in which inactivation of x occurs is complex. . In humans the process involves methylation and modification of the chromatin structure of the inactivated X chromosome resulting in a condensed, hyper-chromatinized structure called the Barr body.

This process occurs before the gastrula stage of embryonic development and probably at the late blastula stage when cells are differentiating from pluripotent and totipotent lineages (Strachan and Read 2003). The process is thought to be controlled by two important genes and their regulatory elements: the X inactive specific transcript gene (XIST) and its antisense partner, the X-inactivation-Specific Transcript-Antisense gene (TSIX) (Brown *et al* 1991, Marahrens, 1999). Inactivation is planned and comprises each stochastic and non-stochastic process. Firstly, a "selective advantage" of one sex chromosome mainly x, over the opposite may be a well-described development within the extreme case wherever one sex chromosome is thought to hold a illness gene and seems to be hand-picked against (Migeon, 1998, Luzzatto *et al,* 1979).

This mechanism will operate within the absence of deleterious phenotype or clearly injurious constitution has additionally been shown in cats, wherever the sex chromosome of the Geoffroy cat features a selective advantage over that of the domestic cat (Abkowitz *et al,* 1998). Secondly, as a result of its believed that somatic cell mainly stem cell division might stochastically end in zero, one or two daughter offspring stem cells, many stem cells might become overrepresented or may be underrepresented by chance (Abkowitz *et al* 1996, Christensen *et al* 2000). Thirdly,

enlargement of a clone derived from one somatic cell mainly stem cell or their precursor might occur with a proliferate advantage (cell selection) (Vickers *et al,* 2001) and therefore by overrepresented. Fourthly, mutations or polymorphisms affecting the control of X-inactivation such as those occurring at the X- inactivation centre or the X inactive specific transcript gene may skew inactivation ratios (Naumova et al 1996).

All of these proposed mechanisms lead to a somewhat confusing To prove definitively that X-inactivation is responsible for an observed phenotype would be difficult and would require some knowledge of how a phenotype is caused (including which cell population or tissue is responsible), whether the abnormal phenotype causes cell selection and what the tolerance to abnormal gene product levels are. Similarly, it would be necessary to show that the tissue sampled, is indeed responsible for the phenotype. Usually this can be owing to the observation that though in normal females X-inactivation ratios square measure often similar between tissues (Sharp *et al* 2000), in many condition of x linked complete skewing is also restricted to specific cell lineages and completely different patterns of X-inactivation seen in all different tissues (Belmont, 1995).

The x linked gene may also escape x inactivation to some extent an upto 15% and also the proportion become different between different area of x chromosome (Carrel et al 1999, Carrel and Willard, 2005) reflective the past and evolutionary history of the sex chromosomes. an extra 10% of sex chromosome mainly x linked genes show different order of inactivation and expressed to completely different extents from some inactive X chromosomes (Carrel and Willard, 2005). Therefore, the degree of X-linked gene expression heterogeneity among females

is great and complex. Methods of assessing X-inactivation and its contribution to phenotype are therefore accompanied by an array of important caveats.

Mellott et al., (1999) provides a giant four-generation pedigree separating sex chromosome mainly x linked conginetal nystagmus and deuteranomaly or inexperienced visual modality defect .he studied large number of members of this family which are 65. Among of these 65 members, thirteen members are affected with conjugate horizontal nystagmus having pendular or jerk waveforms. Many late members of the family were conjointly determined to be affected with the same case which is known by examination of medical records. Some member had gently reduce visual sense conjointly however nobody had important ocular or neurological abnormalities. Eighteen people were conjointly found to be deuteranomalous trichromats, having five girls a minimum of one feminine is also a carrier for visual modality defect and nystagmus.

Had one son suffer also with deuteranomaly seaparetly and a second one transmissible each Condition. The examination of linkage was performed by Mellott et al.,(1999) and also noted that scintist Rucker (1949) had reportable a family with sex chromosome mainly x linked nystagmus during which a person having nystagmus and associate degree red-green colorblindness had two affected daughters and an affected grandchild Oh et al., (2007) reportable five diseased members from three Korean families having sex chromosome x linked conginetal nystagmus .Histories of nystagmus from birth in all are present and having congugat ocular oscillations bilateral and no impairment within the affrent visual pathways. Elaborated reports of the primary family mention that a six-year-old child had conjugate,

horizental and pendular nystagmus major within the primary position. The nystagmus changed into jerky nystagmus throughout lateral gaze. .various type of nystagmus may be appeared during leftward gaze in which Left-beating, upbeating, and counterclockwise torsional nystagmus appeared throughout , whereas right-beating, upbeating, and right-handed or clockwise torsional nystagmus occur throughout rightward gaze. Optokinetic nystagmus direction is also inverted. Such type of continuous eye movements didn't cause oscillopsia, problem of reading, or giddiness. His mother which are 39-year-old had also abnormal eye movements and head tilt rightward head since age one year.

Examination of ocular showed that the nystagmus and right exotropia with clockwise, dowenbeating and left-beating, torsional parts within the primary position. Nystagmus was upbeating and right-beating, and also right-handed or clockwise torsional throughout rightward gaze, and may also downbeating and left-beating, and counterclockwise torsional throughout leftward gaze. Type optokinetic nystagmus direction should be opposite in each patients. No complained of giddiness. Some members of the family had additionally abnormal ocular oscillations. In The second family brothers are affected, and also affected male first cousin, associated an additionally affected maternal grannie. The third family additionally had some diseased membersin both male and female. Eye-movement defects may be completely thought by gaze evoked nystagmus, pendular and jerky oscillations, less or completely absent sleek pursuit and less or completely absent vestibuloocular reflexes.

Alternative feature enclosed increased rate waveforms, mostly foveation phases, direction modification with gaze shift and also reversed optokinetics. The pattern is usually distinct, even

within the same family. Some patients had gently reduced visual modality. Penetrance among feminine carriers was regarding 50%. Thomas et al., (2008) compared ninety patient clinical options having nystagmus disorder attributable to FRMD7 mutations with forty eight patient having nystagmus but not due to FRMD7 mutations. There have been no variations in mean sharp-sightedness or visual acuity and abnormal condition of strabismus between the two groups; most had sensible and good acuity and also stereopsis. Abnormal head condition was considerably more within the non-FRMD7 class.

Pendular nystagmus was additional and more common within the FRMD7 class. Nystagmus amplitude was additional powerfully depend in to the direction of gaze within the FRMD7 class, and are lower at primary and beginning position compared to the non-FRMD7 class. Fifty three percent of obligate feminine carriers of FRMD7 gene mutations were affected. Periodic alternative nystagmus (PAN ;) this nystagmus is spontaneous with a sinusoidally modulated amplitude and also periodic spontaneous changes in direction (Huygen et al., 1995). Huygen et al., (1995) rumored a mother and girl which is his daughter with inborn or congenital beginning of periodic alternating nystagmus. Each had developed offsetting abnormalcy. Case history disclosed several affected members of the family, and therefore the pedigree pattern was per X-linked dominant inheritance. The primary generation contained eleven affected female.

Ito et al., (2000) rumored a lady having alternating periodic nystagmus whose mother had inborn or fixed congenital nystagmus. In the girl is noted intermittent oscillopsia at eighteen years of ages. Study tells us that horizontal spontaneous nystagmus reversed its direction frequently on primary gaze. Sleek pursuit was additionally impaired. Her mother had also show pendular and jerky nystagmus on primary and lateral gaze. Sleek pursuit was additionally impaired. Ito et al. (2000) noted the likeness between the two patients and urged that the daughter's nystagmus was possibly come from birth, even if it absolutely was not detected till later. The authors urged that the two disorders share a standard common underlying mechanism.

Hertle et al.,(2005)also outlined periodic infantile alternating nystagmus as almost like infantile nystagmus , but the difference between two are the null point shifts position during a cyclic pattern. This ends up in periodic or aperiodic changes within the two which are amplitude and direction of the nystagmus each couple of minutes or seconds.

Hertle et al. (2005) also represented the clinical and electrophysiological characteristics of four members of the family from three generations WHO had sex chromosome mainly x linked periodic infantile alternating nystagmus. in two generations three males and one feminine were examined. Clinical tests clue that a jerk-pendular nystagmus with a latent part, strabismus, and a major refractive error within the three affected males, and solely myopic astigmatism within the feminine.

All four members of the family showed eye movement recording (EMR) abnormalities with infantile jerk/dual jerk and pendular nystagmus waveforms. The feminine had nystagmus gift on EMR solely, and every one patient showed regularity to their nystagmus. Mapping studies weren't performed. within the family according by Hertle et al., (2005), Thomas et al.,(2011) known a mutation within the FRMD7 gene.

Khan et al., (2011) describe a family with two brother with sex chromosome x linked infantile nystagmus and three well females having no symptom WHO had delayed the corrective saccades throughout optokinetic nystagmus (OKN) drum testing. All of those people carried a mutation in FRMD7. A maternal aunty had also infantile nystagmus additionally to inborn pathology or conginetal fibrosis of the extraocular muscles (CFEOM). She failed to carry the FRMD7 mutation or a mutation in any famed CFEOM genes, and Khan et al.,(2011) complete that her nystagmus delineated a second disorder during this family, probably associated with CFEOM.

Waardenburg (1962) expressed that there was no cause to segregate x linked dominant frm recessive type kind as some have tried .most of families have dominant in one series and recesive in other (Hemmes, 1924; Waardenburg et al., 1961). The reason can be that the mutation is identical however that a series of 'wildtype' isoalleles have totally different effects on penetrance of the mutation within the heterozygous feminine.

Chapter 3

MATERIALS AND METHODS

Present Study involves two indispensable phases, i.e.; Field Work and Lab Work.

3.1 Field Work

3.1.1 Identification and Enrollment of Families

Family was visited at their local town to meet affected individuals as well as elder members to get detail information about the disease status and after this pedigrees were drawn. The family had other relatives affected with eye disorder and they were also included in the study depending On their willingness and availability. Informed consent form (Figure 3.1) was obtained for participating in this study.

Each family member are interviewed to get complete information about disease and also to confirm the presence of other disorders. Family members were questioned about numbers of patient in family, their history, numbers of generation involved, problems relating to vision and infectious disease like meningitis, antibiotic usage and injury.

3.1.2 Pedigree Drawing and Analysis

The pedigree was constructed on the basis of information collected from the elder members of the family. Bennet *et al., (1995)* described the standard method for drawing the genetics pedigrees so pedigrees were drawn for families according to this method. Male members are shown by square and females shown by circle. Filled circles and squares showed the affected members while unfilled circles and square representing normal individuals. Dead members are representing by a slanting line on the circle or square. Every generation is symbolizing by the Roman numerals while individual with in a generation is symbolized by the Arabic numerals. The consanguineous marriages are represented by the double lines. Pedigree of the families can help in identifying the mode of the inheritance of the disorder. The pedigree was later on constructed electronically in Cyrillic® program on computer.

3.1.3 Blood Sampling

Samples of blood was taken from family members from affected and 2 to 3 normal individuals along with their parents in a clean 5ml sterilized syringes while butterfly needles are used for children less than 2 year of age. These blood samples from the syringe were then placed in EDTA tube (BD, USA). Then these blood samples were stored at 4°C during transportation to research. However, blood was frozen at -20°C in the laboratory until used for DNA extraction.

3.2 Lab Work

Lab work includes electronic pedigree drawing, DNA isolation from blood and quantification, selection of candidate genes for sequencing, primer designing and synthesis for DNA sequencing, PCR amplification of sequencing primers, DNA sequencing and DNA sequence analysis.

3.2.1 DNA Isolation

DNA was separated from blood samples following non-organic method (Grimberg et al., 1989). Samples of blood were thawed for the red blood cell lysis. Washing/T.E buffer (10mM Tris HCl pH8, 2mM EDTA) was added to each sample so that total volumes reach up to 40ml in falcon tubes. Then these samples were centrifuged at 2700 rpm in the Beckman TJ-6 Centrifuge for 20 minutes at 20°C.after 20 minute Supernatant was removed and then basal pellet was broken gently by tapping. Repeat washing for 3 to 4 times till the WBCs' pellet got free of hemoglobin (RBCs). The pellets were resuspended in proteins' digestion mixture containing 6ml of TNE/A1 buffer (10mM Tris HCl pH8, 2mM EDTA, 400mM NaCl), 50 µl of Proteinase K (10 ug/µl) and 200 µl of 10% SDS (each for 10ml of blood volume). The samples were left overnight in an incubating shaker at a temperature of 37°C and a speed of ~250 rpm.

Proteins were precipitated by adding 0.1ml of 6M NaCl per ml of blood volume, followed by vigorous shaking and chilling on ice for 15 minutes. The samples were centrifuged at ~2700 rpm for 15 minutes at 20°C. Supernatant was taken out in a separate tube and centrifuged as in previous step. Supernatant was taken out in a separate tube and the protein pellet was discarded. DNA was precipitated in the form of white threads from the supernatant by gently inverting the tubes after adding equal volume of isopropanol. After 10 minutes samples were centrifuged at ~2700 rpm for 10 minutes at 20°C. Supernatant was discarded and 10ml volume of 70 % ethanol was added to wash the pellet. Samples were again centrifuged as in previous step. Supernatant was discarded carefully and pellet was dried at room temperature. DNA was dissolved in 1.5ml (for each 10 ml of blood) of autoclaved TE for DNA (Tris 10 mM, EDTA 0.1 mM). The samples were left overnight in an incubating shaker at a temperature of 37 °C and a speed of ~250 rpm to dissolve the DNA. Heat shock was given at 70 °C in a shaking

water bath for 1 hour to inactivate any remaining nucleases. All the samples were transferred into properly labeled small screw capped tubes.

3.2.2 Quantification of Isolated DNA Samples

DNA samples were quantified by agarose gel electrophoresis and Optical Density (OD) measurement.

3.2.2.1 Agarose Gel Electrophoresis

This method uses the UV-induced fluorescence of Ethidium Bromide dye intercalated into the nucleic acid. The amount of fluorescence is proportional to the amount of nucleic acid present. Fluorescence of the test DNA and that of a standard DNA was compared. 1.0 % Agarose gel slab (stained with 6µl (10µg/µl) Ethidium Bromide) was prepared. Gel was loaded by 2µl of each DNA sample mixed with 4 µl of loading dye (Bromophenol blue 2X) and was run on 120 V for 35 minutes. The gel slab was observed in gel documentation apparatus (UV trans-illuminator) by using Grab IT software. The samples' quantity was estimated and recorded in DNA extraction sheets. All the DNA samples were stored in IIUI repository at -20 °C until processed further.

3.2.2.2 Optical Density Measurement (Spectrophotometery)

DNA concentrations were obtained by measuring the optical density (OD) at 260 nm using Nanodrop™ spectrophotometer (Table 2.1). The ratio of readings taken at 260 nm and 280 nm wavelengths indicates of the purity of the nucleic acid. DNA quality measurement is based on the fact that absorbance (A) at 260 nm is twice that at 280 nm if the solution contains pure DNA. If there is a contaminant like protein, there is some additional OD which decreases the absorbance ratio between 260 and 280 nm. Pure nucleic acid samples would have an A260/A280

ratio of 2.0. Ratios less than these indicate contamination of protein and the estimates of DNA concentration would be inaccurate.

3.2.3 Candidate Gene Selection

Instead of linkage analysis, direct sequencing approach has been used during this study. For this purpose, selection of the candidate genes was based on four approaches:

(a) **Clinical analysis** of the pedigrees of x linked nystagmus was used to rule out the possibility of all other genes reported for other types of nystagmus.

(b) **Mode of inheritance** was checked during pedigree analysis to identify the mode of inheritance. Certain types of nystagmus only exhibit themselves in a particular mode of inheritance. This was used to further strengthen the candidate gene analysis in conjunction with the pathological conditions.

(c) **Previous research work** was used to identify the relationship of clinical analysis and mode of inheritance with the possibility of finding causative genes in a certain pedigree.

(d) **Exons with most mutations** were searched from the reported mutations from different world populations for nystagmus genes. Only the exons which are having most of mutations reported for a certain type of nystagmus confirmed by clinical analysis and mode of inheritance with the help of reported literature.

3.2.4 Designing of Sequencing Primers

Sequencing primers were designed after selection of candidate genes and their most plausible candidate exon selection. Primers were designed using Primer3 web server (http://www-genome.wi.mit.edu/cgibin/primer/primer3_www.cgi) for these two genes. The virtual performance of designed primers against human genome were checked by In-Silico PCR utility

(http://genome.ucsc.edu/cgi-bin/hgPcr?command=start) and BLAT search (http://genome.ucsc.edu/cgi-bin/hgBlat?hgsid=345100591&command=start) tools of UCSC Genome Browser. Finally, the selected primers were synthesized by Invitrogen, Carlsbad, CA, USA.

Overview of primer of nystagmus causing gene FRMD7

Pairs	Primer ID	Base Count	Primer Sequence (5'-3')
1	FRMD7_Exon1-F	22	GCCCTTGTGAAATAAAGAAAAT
	FRMD7_Exon1-R	24	TGGAAAAATATGAACTACATTGTG
2	FRMD7_Exon2-F	22	AAATTTGCAGATGTAGCCATAG
	FRMD7_Exon2-R	22	TAATAAACAAAGAGGGAGGACA
3	FRMD7_Exon3-F	22	AGATCAACAATGTTCACTGTCA
	FRMD7_Exon3-R	20	ATTGCAGATTGACATCAAGG
4	FRMD7_Exon4-F	21	AAATAAACTCGAAGGCAGAGA
	FRMD7_Exon4-R	22	CAGGCTTGAACTTCTAGAACAC
5	FRMD7_Exon5-F	21	CCTCCTGAGTGCATTTATTCT
	FRMD7_Exon5-R	21	TTAATCCACAGAACCAAAGGT
6	FRMD7_Exon6+7-F	20	CTACAAAGAAGGGGCTGTTT
	FRMD7_Exon6+7-R	21	TATGATTGACCATTTCCCTTT
7	FRMD7_Exon8-F	20	CCTTTCCTCTTGTGATCTCC
	FRMD7_Exon8-R	21	GCCCCATTTTCCTATTACTTT
8	FRMD7_Exon9-F	21	GGTATTTAAGGTGAGGGGAGT
	FRMD7_Exon9-R	21	CTCTGATCCTACCCCTTAGGT
9	FRMD7_Exon10+11-F	21	TACCTGCGTTCTGAGTAGTTG
	FRMD7_Exon10+11-R	21	TGCCAGTTCTCTCCAGTCTAT
10	FRMD7_Exon12a-F	21	AGTAGGATGGCATTGAGAAAG
	FRMD7_Exon12a-R	21	TCCTTTCCTCTGCTCTAATTG
11	FRMD7_Exon12b-F	21	CAGCTAACTTACACGGATGTG
	FRMD7_Exon12b-R	21	AGCAGTTGGTGTGTTGAAATA
12	FRMD7_Exon12c-F	21	ATGGCAAAGAAATAAGGTCAC
	FRMD7_Exon12c-R	22	CCATGCTCTGCTTTTATATGTT
13	FRMD7_Exon12d-F	21	TGTGTTGATCTTTGTGCTCTT
	FRMD7_Exon12d-R	21	AAAATGGAATTTTGGTTCATT

3.2.5 DNA Sequencing (by Sanger Dideoxy Chain Termination Method)

3.2.5.1. Polymerase Chain Reaction (PCR)

Polymerase chain reaction was performed in 0.2 ml tubes (Axygen, USA) containing 20 µl total reaction mixture in (Table 2.1 for PCR mixture).

Table 3.1: PCR Reaction Mixture

Ingredients	Final Conc.	Stock Conc.	Required Volume
Genomic DNA	50 ng	25 ng/µl	2 µl
Primer Forward	0.5µM (5pmol)	10µM	1 µl
Primer Reverse	0.5µM (5pmol)	10µM	1 µl
dNTPs (dATP,dTTP,dCTP, dGTP)	0.2mM (200µM)	2.5 mM	1.6 µl
PCR Buffer	1x	10x*	2 µl
Magnesium Chloride	2.0mM	50mM	0.8 µl
Taq Polymerase	1 units	5 units/µl	0.5 µl
dH_2O	-	-	**q.s to 20 µl

* 10X PCR buffer (100 mM Tris Cl-pH 8.4, 500 mM KCl, and 1% Triton)

** q.s = quantity sufficient

The reaction mixture was centrifuged for few seconds for careful mixing. The reaction mixture was taken through thermocycling conditions which are mentioned in the table 2.2. The PCR was performed by the using BIO RAD T100™ thermal cycler.

Table 3.2: PCR Reaction program.

Steps	Cycle	Time	Temperature
Initial denaturation	1 x	05 minutes	95°C
Denaturation	35 x	60 seconds	95°C
Annealing		45 seconds	55-62°C
Extension		2 minutes	72°C
Final Extension	1 x	10 minutes	72°C

3.2.5.2. Agarose Gel Electrophoresis and Exo-SAP Treatment

5 µl of the PCR product was analyzed on a 1.5% agarose gel to confirm the amplification and to check the purity of the amplicon before sequence analysis. The DNA was then treated to remove unincorporated nucleotides and oligonucleotides with a mixture containing exonuclease-1and shrimp alkaline phosphatase (SAP) (Table 3.3).

Table 3.3: Reaction mixture for Exo-SAP treatment.

Ingredients	Required Volume
Amplified DNA	10 µl
Exonuclease-1	0.2 µl
Shrimp Alkaline Phosphatase (SAP)	0.2 µl
dH_2O	q.s to 15 µl
Incubated at 37oC for 1 hour, followed by 80oC for 15 min to inactivate the enzymes and	

3.2.5.3. Sequencing Reaction

To the above 15 µl reaction, equal volume of dH2O was added to dilute the salt concentration in the samples which otherwise could affect the sequencing results.

Sequencing PCR (Fig. 2.1) either with forward or reverse primer was performed using the following reaction mixture (Table 2.4).

Table 3.4: Reaction Mixture for sequencing reaction.

Ingredients	Required Volume
Diluted Amlicon after Exo-SAP	6 µl
Big dye sequencing mix	1 µl
Primer (forward or reverse) (3.2pM)	1 µl
5x dilution buffer (Tris-Cl 400 mM-pH 8.7, MgCl2 10 mM)	1 µl
dH_2O	q.s to 10 µl

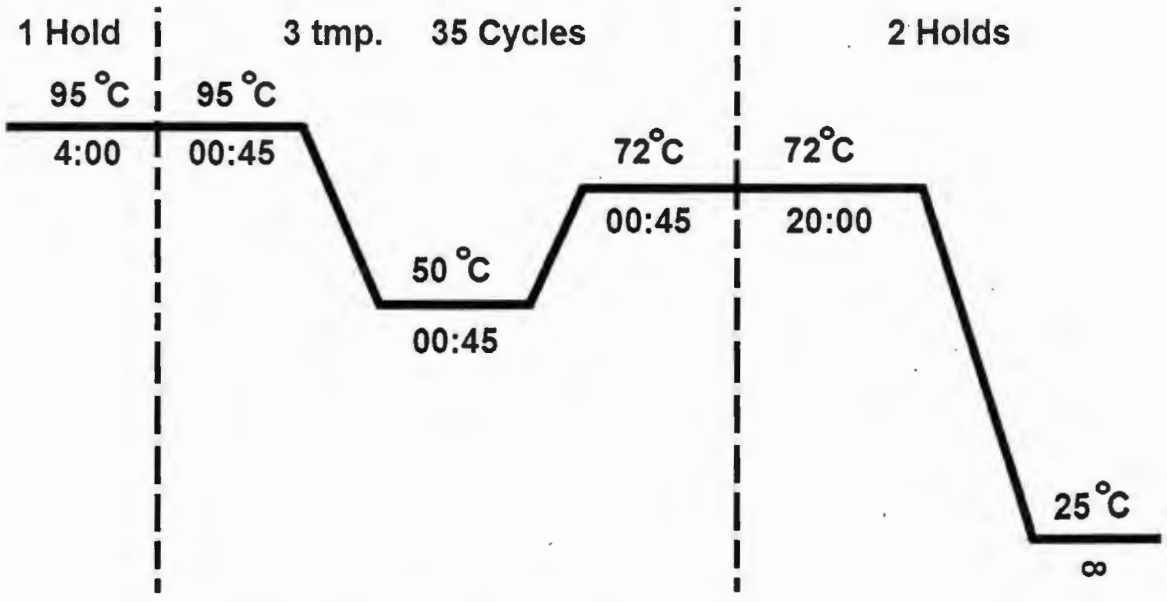

Figure 3.2. Thermocycling profile for sequencing reaction

3.2.5.4 Preparing a Product for Sequencing on ABI Genetic Analyzer

Sequencing reaction was set up in 0.2 ml PCR tubes and precipitated using ethanol. 19.0 µl of 95% ethanol and 1.0 µl of dH2O were added to the 10 µl sequencing reaction to make the final concentration of ethanol up 60± 3%. PCR tubes were inverted a few times to mix and were kept at room temperature for 15 min to precipitate the extended products. The tubes were centrifuged at 3250 rpm for 30min and supernatant was discarded. 40 µl of 70% ethanol was added to each tube to rinse the pellet, and the tubes were again centrifuged at 3250 rpm for 20 min after. Finally ethanol was discarded similarly as above, pellets were air dried and dissolved in 12 µl of deionized Hi-Di™ Formamide (ABI). Samples were denatured at 95°C for 5 min and quick chilled by placing in ice before loading on the ABI Genetic Analyzer.

3.2.6. Analysis of DNA Sequences

Sequencing data was analyzed manually by using Chromas software (v1.45). The sequence was also blast against normal sequence by using either Nucleotide-nucleotide BLAST (blastn) or BLAST 2 Sequences on the NCBI web. BLAST (Basic Local Alignments Search Tool) a family of search programs is designed to explore all the available databases against a query protein or DNA sequence. The scores are assigned in a BLAST search based on a statistical interpretation, thus differentiating real matches from random background hits (Altschul et al., 1990).

3.2.6. Phylogenetic Analysis

Phylogenetic analysis of FRMD7 gene (nucleotide and peptide sequence) was performed using CLUSTALW web based application (www. Genome.jp/tools/clustalw/). Sequence data was provided in a single file in FASTA format.

Chapter 4

RESULTS

During field work, six families with history of nystagmus are identified belonging to province Khyber Pakhtunkhwa and three different districts bunner, Karak and Peshawar of Pakistan was identified. Among these families only one family is x-linked recessive (Fig: 4.1) while the other five families are autosomal. These families were included in the study with the prior consent of all the family members. Blood samples were taken from different family members affected as well as normal for isolation of DNA which was used for their genetic analysis. This family was also subjected to clinical analysis for confirmation of disease status.

Table 4.1: Pedigrees information about PKNYS-09 family.

1	PKNYS-09	Pakhtun	Khan	Peshawar

4.1 Family PKNYS-09

4.1.1 Family History

This family was enrolled from Peshawar city. It has seven affected individuals. All alive affected individuals have shown symptoms of nystagmus from the beginning of their life. This family was identified as carrying nystagmus with X-linked pattern of inheritance.

Various other symptoms along with nystagmus are also included such as low vision and albinism. Pedigree analysis shows that it is x-linked recessive family.

4.2 Clinical findings

In this family totally seven alive individual are affected out of which two are female and five are males. Skin and hair pigmentation are also affected of mostly family member and six out of seven are affected with albinism. Most of family patients are males and they show involuntary movement of eye ball without head nodding. All family members with nystagmus have reduced vision. Nystagmus symptoms starting from birth and also show symptom of involuntary movement of eye ball in later life. Sometime the involuntary movement of eye become severs with the passage of time. At the age sometime nystagmus is cured but sometime it becomes sever with age.

4.3 Pedigree of PKNYS- 09:

Hand drawn pedigrees were finally drawn with the help of Cyrillic software (Figure 4.1). Circles are males whereas squares are females. Horizontal line joining the two symbols is a marriage line whereas vertical lines emerging from the marriage line indicate the offspring line. Filled symbols represent affected individuals and unfilled are normal. Diagonal lines on any symbol indicate deceased individuals.

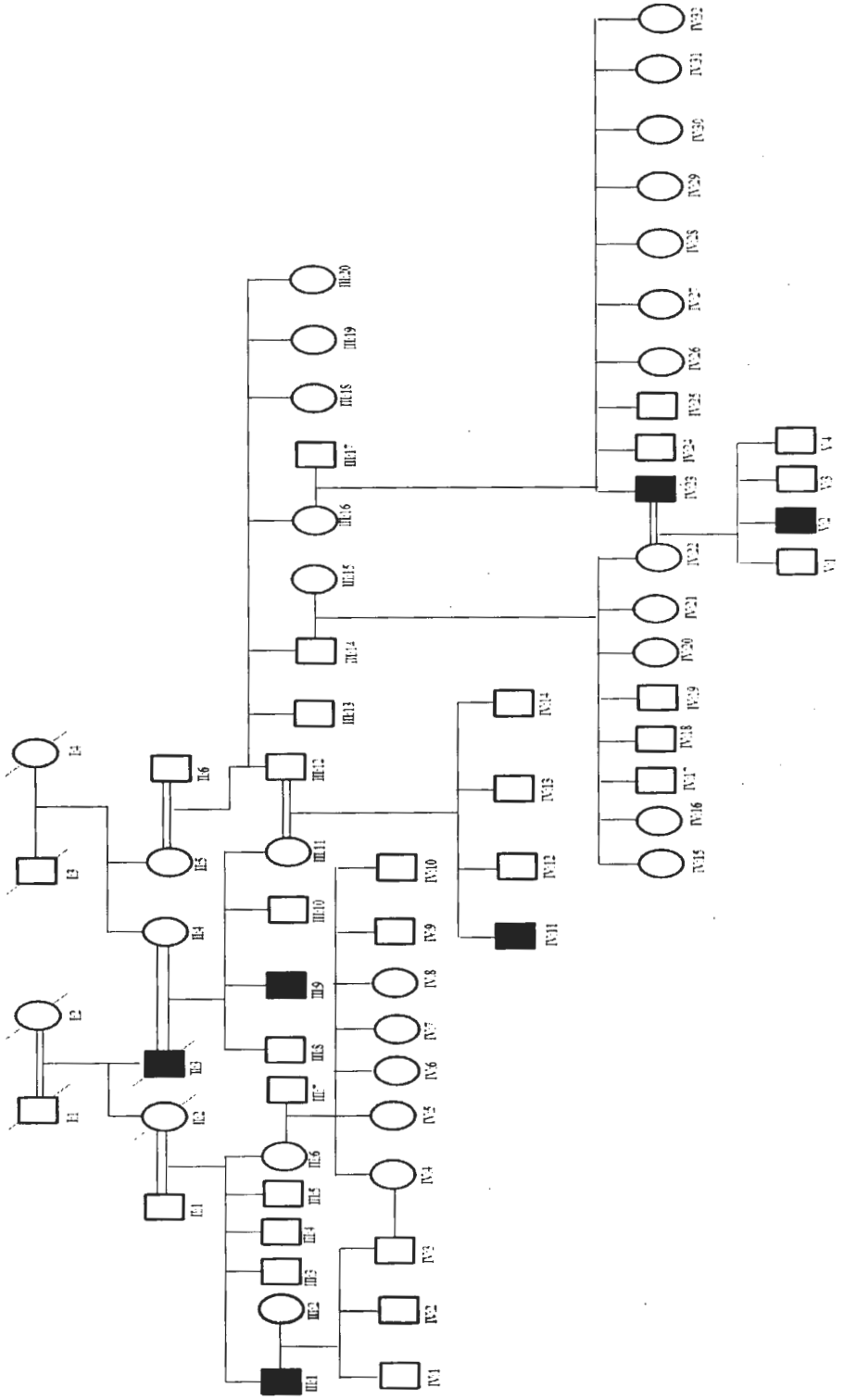

Figure 4.1. pedigree of X-linked family PKNYS-09

Other Families:

Various other families are also collected during field work but their analysis show that they are autosomal and our work related to X-linked. Only single family analyses show that it is X-linked recessive. So this single family is selected for further work. The pedigrees of all other families are also drawn below.

PKNYS-03

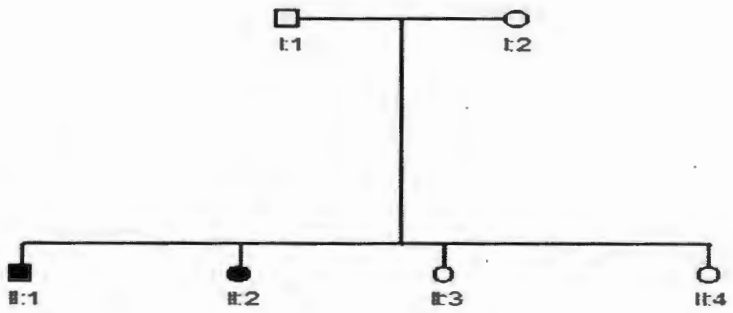

Figure 4.2. pedigree of autosomal family PKNYS-03

PKNYS-04

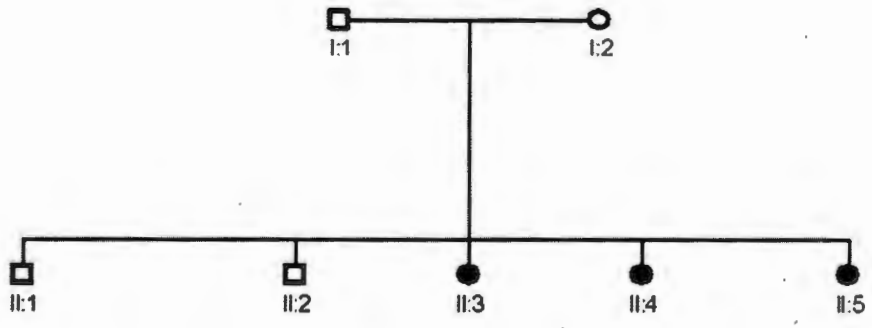

Figure 4.3. pedigree of autosomal family PKNYS-04

PKNYS-05

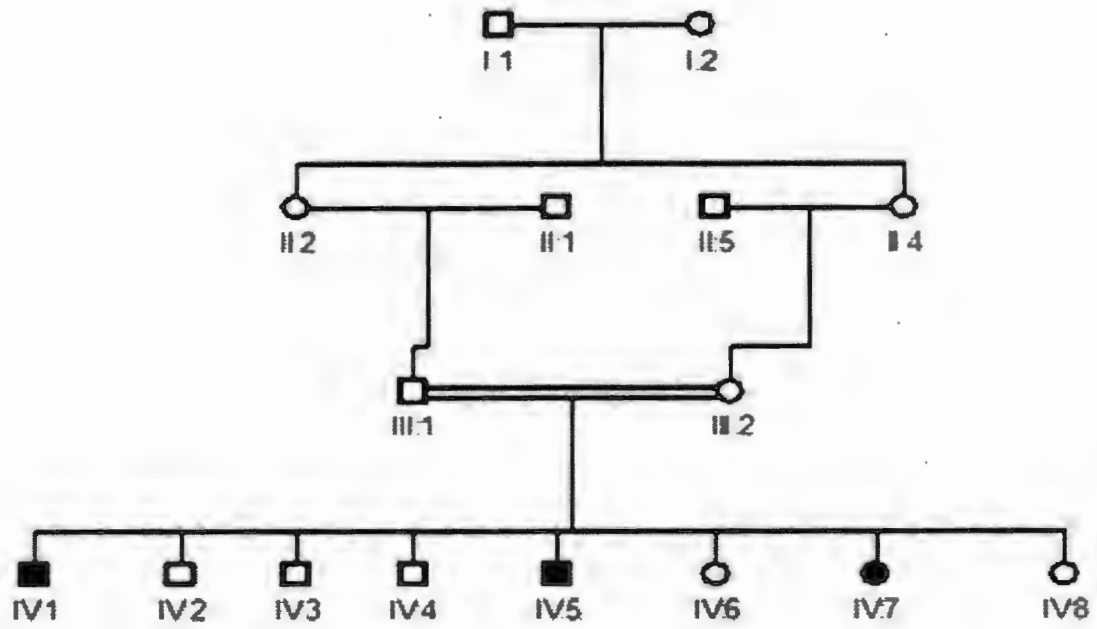

Figure 4.4. pedigree of autosomal family PKNYS-05

PKNYS-06

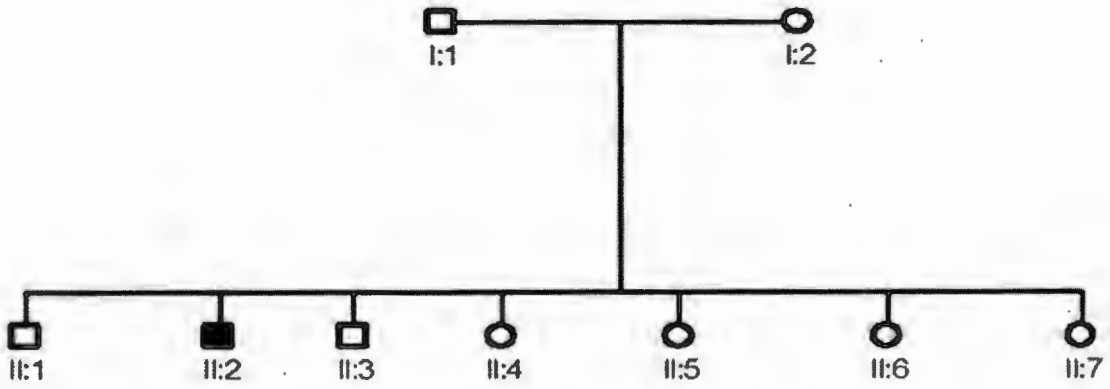

Figure 4.5. pedigree of autosomal family PKNYS-06

4.4 DNA Isolation and Purification

DNA was extracted from blood by standard non-organic method as described in Chapter#10 material and methods. DNA was isolated from blood which is collected from patient as well as from normal.

4.4.1 Quantification of DNA

The presence of isolated DNA was confirmed by agarose gel electrophoresis (Fig.13). For quality and quantity of the isolated DNA, UV/Visible spectrophotometry was performed using Nano drop Spectrophotometer and the results are shown below in the form of a table (Table 3.2).

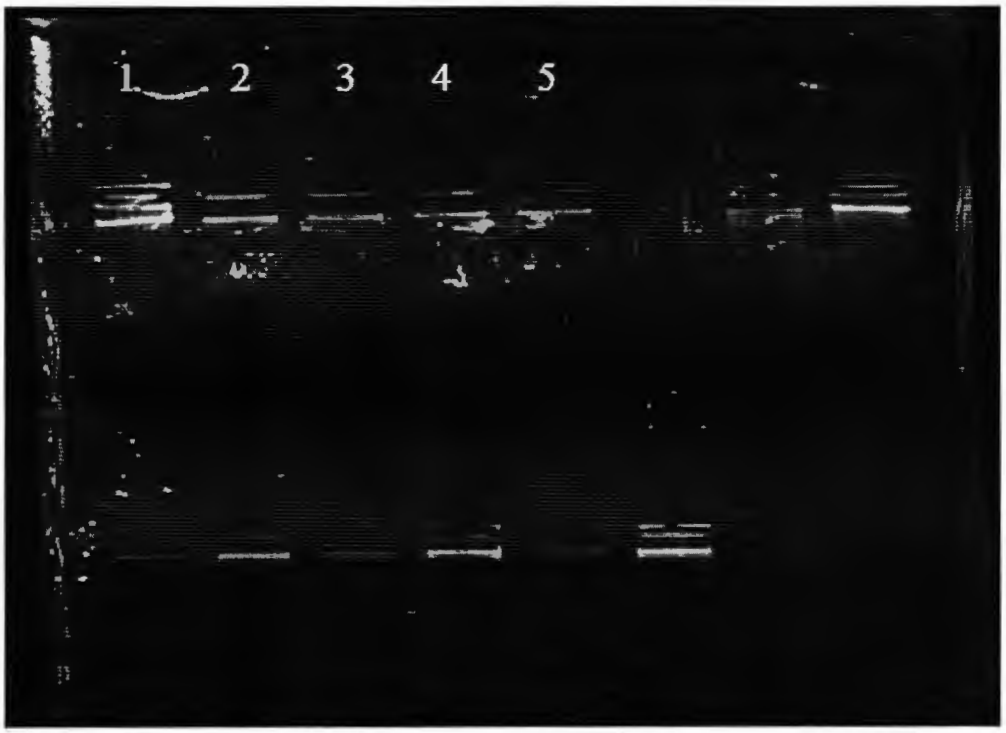

Figure 4.6. Genomic DNA from different family members from PKNYS-021 on 1% agarose gel

Table 4.2: Nanodrop Spectrophotometry results for family PKNYS-09

No.	Individual ID	DNA conc. (ng/ul)	A_{260}	A_{280}	260/280	260/230
1	III:1	181.9	3.639	1.923	1.88	1.65
2	III:9	156.3	3.126	1.654	1.86	1.46
3	IV:11	127.8	2.555	1.435	1.78	1.12
4	IV:23	158.6	3.665	1.635	1.87	1.48
5	V:2	2.43.5	6.553	1.987	1.89	1.89

4.5 PCR amplification

4.5.1 Standardization of primers

All the included primers of gene FRMD7 were standardized by using gradient PCR technology. For this purpose BIO RAD T100™ thermal cycler was used. Primers were standardized at different temperatures between 55°C to 63°C.Most of the primers were optimized at 58.1°C.The samples were amplified using first denaturation step at 95°C for 60 seconds, annealed with the primers for 45 seconds at 58.1°C and extended at 72°C for 2 minutesfor 35 cycles. The initial denaturation step at the beginning of the PCR was for 5 minutes at 95°C. A post PCR step for 10 minutes at 72°C was added to extend all unfinished products (Fig.3.5).PCR reaction mixture contained 1X Taq Buffer (10 mM Tris Cl-pH 8.4, 50 mMKCl, 0.1X Triton,), 200 µMdNTPs, 1 unit of Taq DNA polymerase, and 5ρmol of each primer (0.5mM) (Table 3.3).

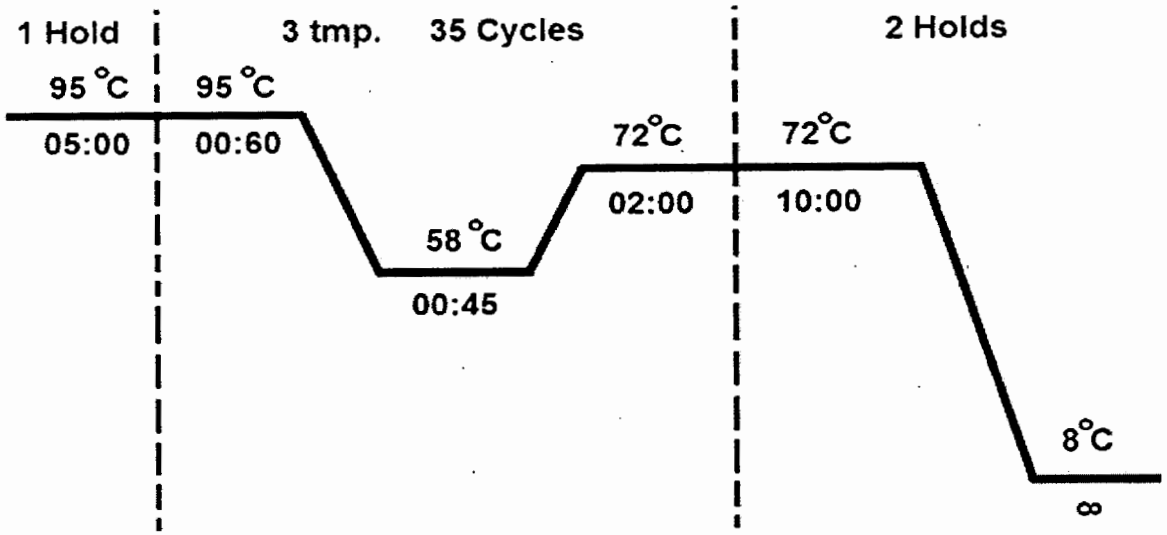

Figure 4.7. A representative PCR program used for amplification of PCR primers.

Table 4.3: Reaction mixture for PCR amplification

Ingredients	Final Conc.	Stock Conc.	Required Volume
Genomic DNA	50 ng	25 ng/µl	2 µl
Primer Forward	0.5µM (5pmol)	10µM	1µl
Primer Reverse	0.5µM (5pmol)	10µM	1µl
dNTPs(dATP,dTTP,dCTP, dGTP)	0.2mM (200µM)	2.5 mM	1.6µl
PCR Buffer	1x	10x*	2µl
Magnesium Chloride	2.0mM	50mM	0.8 µl
Taq Polymerase	1 units	5 units/µl	0.5µl
dH_2O	-	-	**q.s to 20µl

* 10X PCR buffer (100 mM Tris Cl-pH 8.4, 500 mMKCl, and 1% Triton)

** q.s = quantity sufficient

4.6 DNA Sequencing

Three selected exons (9, 10 and 11) from the gene FRMD7 (Figure 4.8.A) that were found the most common cause of nystagmus in world population and were sequenced by Sanger dideoxy chain termination method (Figure 4.8.B). Sequence was analyzed manually by using Chromas software version (v1.45). The sequence was also blast against normal sequence by using either Nucleotide-nucleotide BLAST (blastn) or BLAST 2 Sequences on the NCBI web.

No pathogenic variant or mutation has been observed in this sequenced exon of FRMD7 gene. The details of exons are given in material and methods.

	Intron 8-9	132,084,489	132,082,527	1,963	gtaagtagctttctaataaattcgt.....actttaatggaaaattggtttatacag
9	ENSE00001095575	132,082,526	132,082,363	164	GTGTTGTGGAAGGATACCTTGGAGTTCACCATGGCCAGCCGGAGATGCCTGCAAGGCTTTC TGGAAGACTGGTGTGGAATACCGTGCTTTCTTCAGGCTTTCGGAAGAGCCAAATCAAAG CCCAAAACCTCTCTGCAGGAAGGTTCCGGTTTCCGCTGTAG
	Intron 9-10	132,082,362	132,080,267	2,096	gtaattaaacaatgcacataggtaa.....ggatttttattgttccctatgataag
10	ENSE00001215464	132,080,266	132,080,198	69	TGGAGGAACCCAAAGGCAACTTTTGGAATTTGGAGAAAAGGGAAGCTGAAGAGCTTGTC ATTTGAAAG
	Intron 10-11	132,080,197	132,080,082	116	gtacatgatttgtttctcttgctc.....tgacttcctgcttgggatttaag
11	ENSE00001215459	132,080,081	132,080,006	76	GAAACATTCCCTCTCAGTCCTGAAGACAGTGCAGGTCCTGACCAGACCTCCTCTC TGATGTGTCAAAACAA
	Intron 11-12	132,080,005	132,078,967	1,039	gtaaggtttctgaaaatttctactt.....tcacctaatgtgtccatgctctaag

Figure 4.8.A. Intron/Exon boundaries and sequence for Exon 9, 10 and 11

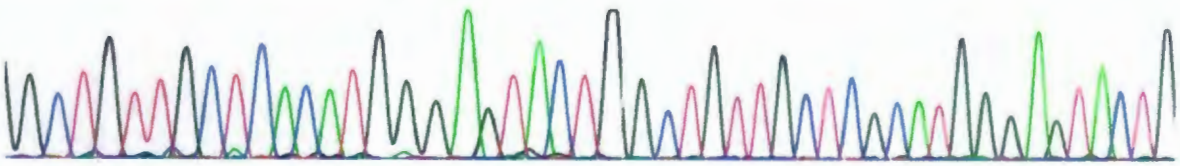

Figure 4.8.B. A representative electropherogram of DNA sequence

4.7. Phylogenetic Analysis (Nucleotide Sequence):

Phylogenetic analysis for the gene FRMD7 was performed using nucleotide sequence from different primates. Those organisms which are close to each other are much similar to each other in nucleotide sequence while those organisms which are far away from each other are less similar to each other in nucleotide sequence. Human and Chimpanzee were found closely related to each other (Figure 4.9).

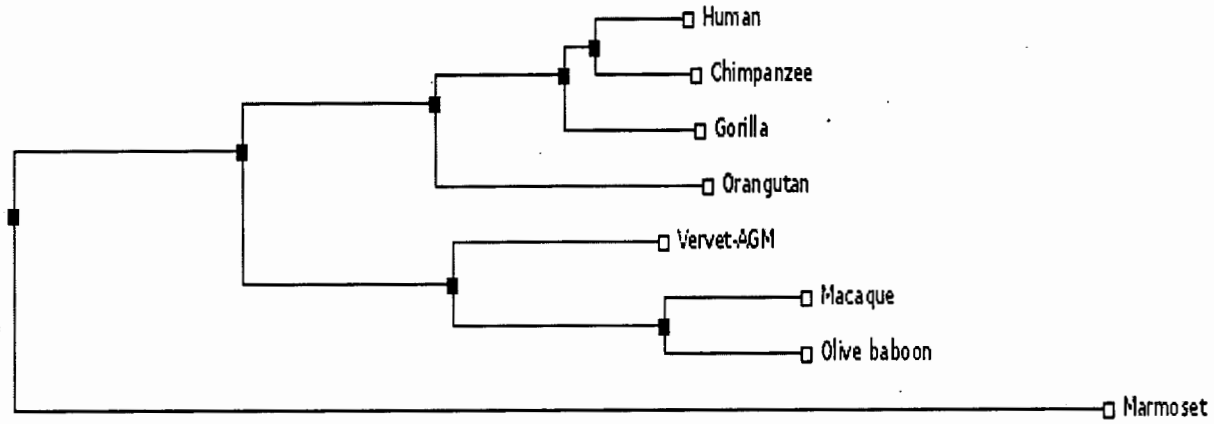

Figure 4.9. Pylogenetic analysis of nucleotide sequence of FRMD7 gene in primates

4.8. Phylogenetic Analysis (Peptide Sequence):

The peptide sequence was also compared in various organisms. Those organisms which are closer to each other are similar to each other while those organisms which are far away from each are less similar to each other. Peptide sequence show phylogenetic relationships on the

basis of peptide sequence conservation. *Homo sapiens* is found closely related to *Pan troglodytes* (Figure 4.10).

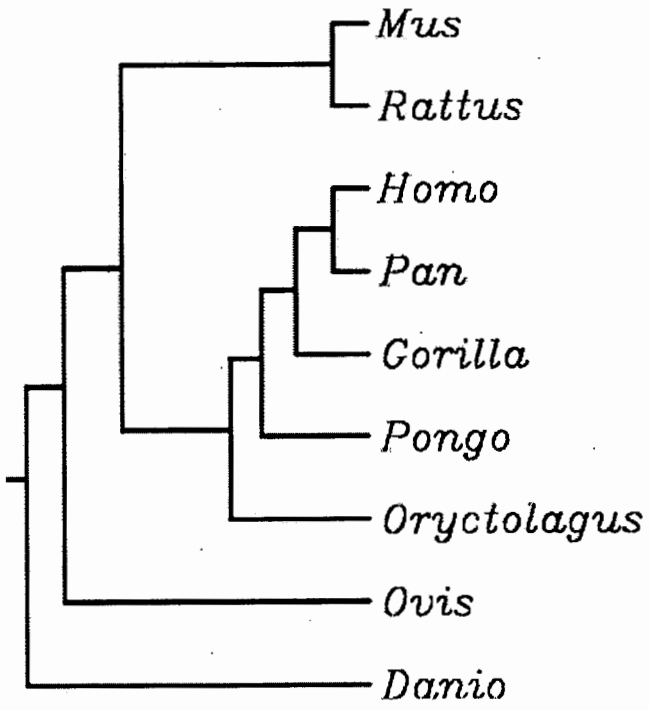

Figure 4.10. Pylogenetic analysis of peptide sequence of FRMD7 gene in different organisms

4.9. Multiple Sequence Alignment (Peptide Sequence):

Multiple sequence alignment of peptide sequence (Figure 4.11) coded by exon nine (Figure 4.12) was carried out for various organisms. Their comparison shows that amino acids that are reported to be affected by different mutations in exon 9 are highly conserved among different species (Figure 4.13).

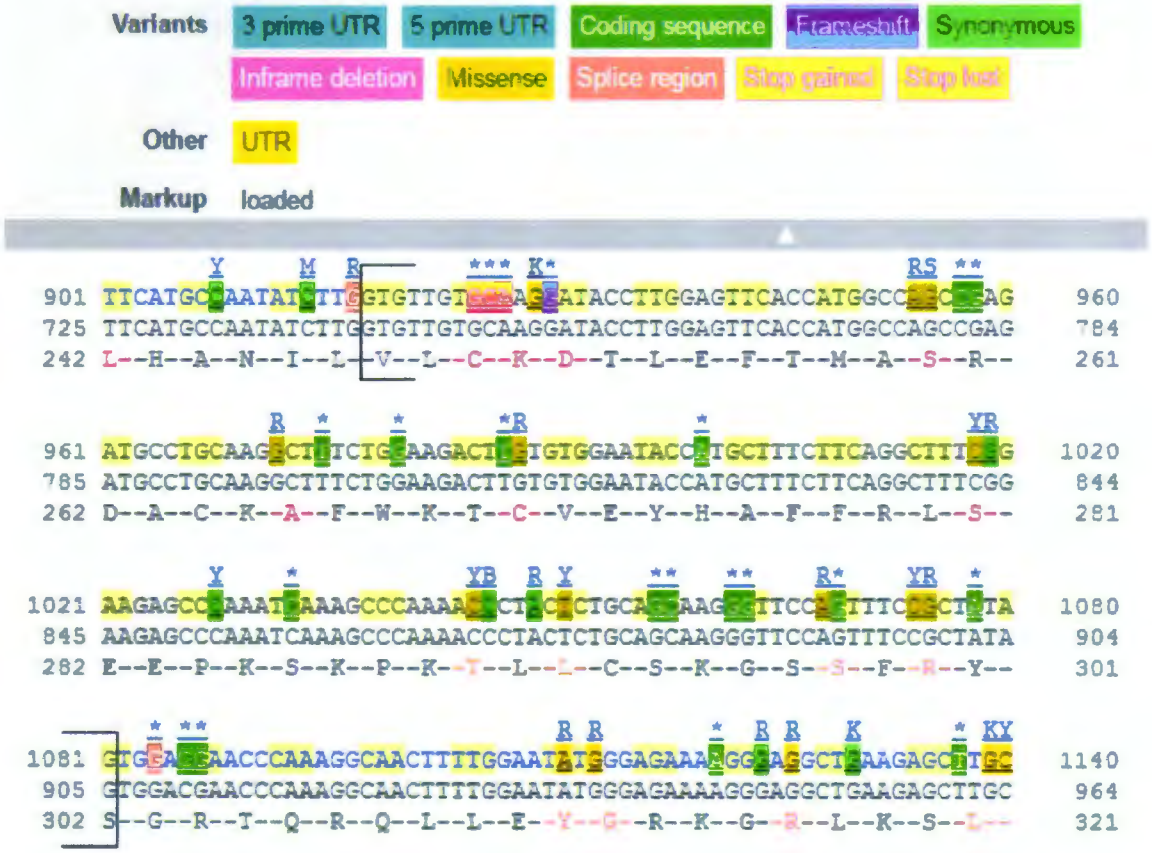

Figure 4.11.Multiple sequence alignment of Peptide sequence coded by Exon 9

Peptide sequence coded by Exon 9 of gene FRMD7 enclosed in big brackets.

This image was taken from ENSEMBL genome browser (http://asia.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g). Red color represent stop gained and stop lose, while the yellow color represent missense mutation. The blue color represent untranslated region at 3 and 5 end. Inside bracket area is selected for multiple sequence alignment. The line below the nucleotide is amino acid line.

Figure 4.12. Nucleotide sequence of Exon 9 of gene FRMD7

Figure 4.13. Multiple sequence alignment of peptide sequence coded by Exon 9 of gene FRMD7

Red arrows represent amino acids that may be affected by different mutations reported in literature

* = represent similarity

: =represent both amino acid of single organism are change

. = represent single change in amino acid

Chapter 5

DISCUSSION

Nystagmus is a condition in which eye movement is involuntary due to which it is sometimes called "dancing eyes". This may be acquired in infancy or later in life and may result in reduced or limited vision. In normal individuals, if the head is rotated about any axis, eyes are rotated in opposite direction to sustain distant visual images are sustained on the respective axis. Angular acceleration is sensed by semicircular canals in the vestibule. Signals from here are sent to the nuclei for eye movement control in the brain. A signal is then relayed from the brain to the Extraocular muscles to allow fixation of one's gaze on one object along with the head movement. If the semicircular canals are stimulated while the head is not in motion, nystagmus occurs. Whichever semicircular canal is stimulated describes the direction of ocular movement being vertical, horizontal or circular (Leigh and Zee, 1999).

Nystagmus is caused by gene named as FRMD7. This gene is composed of 12 exons. The functional region o FRMD7 undergoes mutation and cause nystagmus. There are totally 22 Mutation reported in FRMD7 (Tarpey et al 2006). Total mutation known in FRMD7 is cosegregated with disease in linked families. The X chromosome holds a singular place within the history of medical biological science. Identification of X-Linked disorder is increased by the relative easy knowing this type of inheritance. Moreover, a disproportionately sizable amount of unwellness conditions are related to the X chromosome as a result of the external appearance consequence of a recessive mutation that is disclosed directly in males. This happens as a result of all males is constitutionally hemizygous for any factor having no active counterpart on the Y

chromosome. Thus, though the X chromosome contains solely four percent of all human genes, virtually 10% of disorders with a Mendelian pattern of inheritance are allotted to the X chromosome (307 out of 3,199; info obtained from the OMIM2TM repository (Online Mendalian Inheritance in Man).

Present study was designed to study the molecular players of nystagmus in affected individuals of collected family. Direct sequencing approach was used after identifying the mode of inheritance in the affected family. Most susceptible exons (9, 10 and 11) were sequenced in the collected family; however no pathogenic variant was observed.

During this study, linkage analysis has not been performed; however, direct sequencing approach was adapted. For this purpose, selection of the candidate genes was based on four approaches:

(a) **Clinical analysis** of the pedigree PKNYS-09 clearly indicated the presence of nystagmus.

(b) **Mode of inheritance** in the pedigree PKNYS-09 seems to be X-linked.

(c) **Previous research work** indicates that nystagmus can be manifested in an X-linked fashion and a gene on X-chromosome named FRMD7 is responsible for the disease.

(d) **Exons with most mutations** were searched from the reported mutations from different world populations. Exons 9, 10 and 11 were found to be the most susceptible exons to find any pathogenic variant, however, no pathogenic sequence variant was observed in all these thirteen exons.

There could be several possible reasons for not finding mutations in the selected exons:

Firstly, all the exons were sequenced based on already reported mutations from different world populations and reports on molecular genetics of nystagmus is scarce in Pakistani population.

There is a chance of involvement of some other exon whose mutation is leading to nystagmus in the PKNYS-09 pedigree.

Secondly, some mutation may be present in the 5' and 3' untranslated regions (UTRs) of the gene or may be in promoter regions which were not tested due to shortage of time.

In order to pin-point the molecular reason for the nystagmus pathology in family PKNYS-09, further study is required. If no pathogenic variant is observed in all the exons and the regulatory regions of the gene, there could be the possibility of involvement of any other gene. In such condition, this family can be subjected to whole exome sequencing to find the causative region of the exome. This will further strengthen our understanding of the molecular genetics of nystagmus.

Phylogenetic analysis was performed on nucleotide and peptide sequence of FRMD7 gene. Nucleotide sequence of different primates was subjected to multiple sequence alignment. Human and Chimpanzee were found closely related to each other (Figure 4.9). Similarly peptide sequence of FRMD7 from different species was aligned. *Homo sapiens* are found closely related to *Pan troglodytes ((*Figure 4.10)). Further, Multiple sequence alignment of peptide sequence (Figure 4.11) coded by exon nine (Figure 4.12) was carried out for various organisms. Their comparison shows that amino acids that are reported to be affected by different mutations in exon 9 are highly conserved among different species (Figure 4.13).

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