Identification and Characterization of Genes Involved in Neurodevelopmental Disorder by Whole Genome SNP Genotyping

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FINAL APPROVAL

It is certified that we have read the thesis submitted by Miss Beenish Azad and it is our judgment that this project is of sufficient standard to warrant its acceptance by the International Islamic university, Islamabad, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biotechnology.

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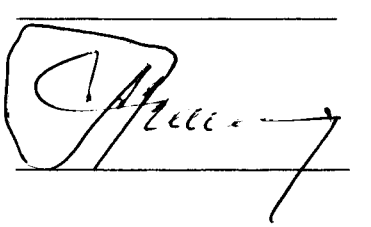

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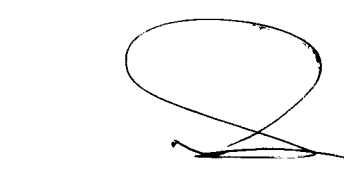

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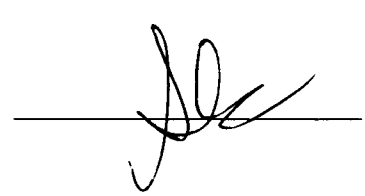

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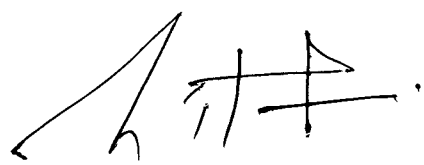

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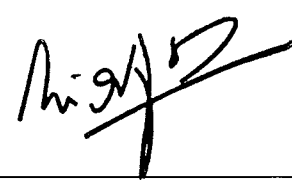

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DEDICATION

This work is dedicated to my beloved parents, my family and my husband whose constant support and guidance enabled me to achieve this milestone.

DECLARATION

It is certified that work done on this PhD Biotechnology research thesis "Identification and Characterization of Genes Involved in Neurodevelopmental Disorder by Whole Genome SNP Genotyping'' is purely conducted by me. All the material mentioned is my own work and has not been copied from anywhere; however, some test and figures have been used which are properly referenced.

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Beenish Azad

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Abbreviations

NCLs	Neuronal Ceroid Lipofuscinoses
INCL	Infantile Neuronal Ceroid Lipofuscinoses
LINCL	Later Infantile Neuronal Ceroid Lipofuscinoses
JNCL	Juvenile Neuronal Ceroid Lipofuscinoses
ANCL	Adult Neuronal Ceroid Lipofuscinoses
NDDs	Neuro Developmental Disorders
CLD	Cell Lysis Buffer
PK	Proteinase K
BBA	Binding Buffer
CWD	Column Wash Solution
EEG	Electro Encephalography
MRI	Magnetic Resonance Imaging
SCMAS	Subunit c of Mitochondrial ATP Synthase
PPT1	Palmitoyl Protein Thioesterase 1
TPP1	Tripeptidyl Peptidase I
LSDs	Lysosomal Storage Disorders
GRN	Granulin
CSPα	Cysteine-String Protein Alpha
CRMP-2	Collapsing Response Mediator Protein 2
Ug	Microgram
Ul	Microliter
KCTD7	Potassium Channel Tetramerization Domain Containing Protein 7

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First, I pay my submissive gratitude in the domain of Almighty Allah who is the supreme authority over the universe, and nothing goes unaccounted under his domain. I am thankful to **Almighty Allah** that he has bestowing me the strength and qualities which enabled me to complete my research.

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ABSTRACT

Neuro developmental disorder is such condition which is associated to development of the brain. NDs typically start during developmental stages, making toddlers, children, and adolescents the most likely to have them. However, they can persist into adulthood or may not be recognised until a person is an adult. Neuronal Ceroid Lipofuscinosis (NCL) is a genetic disease with advanced brain neurodegeneration. The mutation in protein cause different kinds of NCL disease conditions, characterized by visual impairment, motor decline, seizures, and progressive cognitive deterioration. The onset age ranges from early childhood through late adulthood. The onset age ranges from early childhood through late adulthood. This condition is the most frequent neurodegenerative disorder in children with a prevalence of 1:1,000,000 to 1:14,000 in the world. The main purpose of this study was to identify the causative gene associated with NCL in Pakistani consanguineous families. Eleven families under the study were subjected to detailed clinical and neuroradiological characterization. Whole exome sequencing was performed and segregation analysis was done through sanger sequencing.

Whole Exome Sequencing helped to identify two novel homozygous variants c.925_926del (p. Leu309AlafsTer4), and c.477 T > C (p. Cys159Arg) in family A and B, respectively, in the NCL transcript (Neuronal Ceroid Lipofuscinosis 5) and a novel variant, (c.938T>G p. Leu313Arg) in family K, in the MFSD8 gene. This study was conducted with a total of eleven families(A-K), out of which five families revealed mutations in *TPP1* genes. These families showed novel variants for the first time in Pakistan though these variants have been previously reported in other regions of the world. Homozygous mutations in TPP1 were identified with the missense variant ENST00000299427. 12: c.616C>T, p. p. Arg206Cys (chr 11-66,38,277). In two families (D and E) another homozygous mutation in TPP1 was identified with the missense variant ENST00000299427.11: c.622C>T, p. Arg208Ter (chr11-66,38,271) in F family. In families G and H, the homozygous missense variant NM_000391.3 p. Glu402Gly c.1205A>G (chr 1166,36,734) was identified. In CLN6, the family-I missense variant ENST00000249806.11: c.768C>G, (Chr15:68,20,83,08) ENST00000249806.11: p. Ser265del, c.794_796delC (chr1568,500,617) was present.

Our results supported clinical observations and associate common and exclusive clinical features of NCL patients, further refining our present knowledge on NCL. All collected information would help our population for clinical administration, prognosis and family planning with prenatal findings.

1. Introduction

1.1 Neuronal Ceroid Lipofuscinoses

The most common type of inherited neurodegenerative diseases in children is Neuronal Ceroid Lipofuscinosis (NCL), which is a lysosomal storage disorder (Haltia *et al.*, 2003). NCL has been known as one of the most common early-age onset neurodegenerative pathologies, with an occurrence rate of 1:1,000,000 to 1:14,000 worldwide (Gao *et al.*, 2018). Fourteen different types of NCL (CLN1 to CLN14) have been identified (Figure 1.1) (Parvin *et al.*, 2019)., Recently CLN15 a new subtype of NCL has been identified, Except for one adult-onset type caused by mutations in DNAJC5/CLN4 that have been described in autosomal dominant inheritance, almost all of them are inherited in an autosomal recessive way (Panjeshahi *et al.*, 2023).

NCLs have been classified into 6 subtypes such as, congenital, infantile, late infantile, variant late infantile, juvenile, and adult NCL phenotypes (Bartsch & Storch, 2022). In children, neuronal ceroid lipofuscinoses are the most typical cause of dementia. The clinical features in an individual, like loss of vision, gradual repression in cognitive and motor capabilities and epileptic episodes could be the symptoms of NCLs. The onset of these clinical features differs according to the type of the NCLs in every individual (Hemanth *et al.*, 2019). Vision impairment and seizure occur comparatively late in disease course and it is slower in early-infantile and infantile forms compared to other NCLs (Eija *et al.*, 2017). Neuronal ceroid lipofuscinoses can be often misdiagnosed at the onset because of the appearance of non-specific presenting symptoms; therefore, the diagnosis may be delayed (Trivisano *et al.*, 2022). The two main eponyms for NCL at the moment are Kufs disease and Batten disease. Regardless of the age of onset, Batten disease refers to pediatric NCLs, whereas Kufs disease is used to describe the two main phenotypes of adult-onset NCL (Kuf A and B) (Simonati *et al.*, 2022).Patients are classified based on a variety of variables, such as age at onset, the sequence and progression of clinical symptoms, the ultrastructure of storage materials, and the damaged gene (Mole et al. 2005). Neuronal ceroid lipofuscinoses caused by mutations in transmembrane proteins, in comparison, is more challenging. The most important treatment options such as, immunomodulatory therapies, gene augmentation

strategies, or small molecule therapies (Kohlschutter *et al.*, 2019).

1.2 Symptoms

Common symptoms associated with Neuronal Ceroid Lipofuscinosis (NCL) include: progressive vision impairment, ataxia, cognitive decline, epileptic seizures, weakness, loss of acquired motor abilities and spasticity (Mole *et al.*, 2011).

1.2.1 Ataxia

At an age of onset between two and five years, ataxia is the most common clinical symptom of CLN1, CLN2, CLN7, and CLN8 diseases as well as the more uncommon late-infantile version of CLN1 disease (Alessandro and Ruth, 2022).

1.2.2 Cognitive Decline

NCLs are characterised by delays in cognitive and language impairment and in younger children, the loss of cognitive abilities impairs school learning, while behavioural problems include, depressed mood, anxiety, burst of aggressive behavior and psychotic manifestations. During the starting years after disease onset these symptoms remain stable or even worsen (Simonati and Williams, 2022).

1.2.3 Epileptic Seizures

Seizures are one of the earliest symptoms in affected individuals of NCL however the intensity pattern of episodes may vary based on the stage of life: infantile and late-infantile onset NCLs. It has been observed that overtime their frequency dissipates except for myoclonus (Rajesh *et al.*, 2013).

1.2.4 Visual Impairment

Another primary feature of NCL is the progressive visual impairment which has an early childhood onset and it is a common feature in almost all its forms. It affects retinal and cortex structure as well as visual pathways, mutations, however, play a significant effect in defining the degree of severity and even the onset of symptoms in certain age groups. (Alessandro and Williams, 2022).

1.3 Classification and Clinical Phenotype

NCLs has been categorically divided into four subtypes namely infantile (INCL), later infantile (LINCL), juvenile (JNCL), and adult NCL (ANCL) (Table 1.1) (Geraets *et al.*,2016). This nature of categorization helps identify patients who have developed some component of the NCLs in pre-school age through entire exome sequencing. Despite an absence of adequate treatment, early examinations and identification have contributed in helping with maintaining an improved lifestyle for in NCL patients (Kowari *et al.*, 2011). CLN5 disease caused by mutation in ceroid Lipofuscinosis protein 5 which is classified as late-infantile NCL (Table 1.1) (LINCL) because it is severe and rare form of NCL, manifesting between 2 and 8 years of age (Pineda *et al.*, 2005).

1.4 Diagnosis of NCL

Mutation analysis is only key to diagnose the NCL, but exploring other factors such as morphological findings, through the characteristics storage deposits which are unique for each form enable us to understand NCL further (Radke *et al.*, 2015 & Anderson *et al.*, 2013). NCL specific ultrastructure pattern enclose GROD features (curvilinear, rectilinear profiles or fingerprint profiles visible either separate or in combination in other form), the later associating with accumulation of the subunit "c" of ATP synthase (SCMAS) (Cotman *et al.*, 2013).

1.5 Candidate Genes involved in NCL identification

The *CLN1, CLN2, CLN3*, were identified first time in 1990s. Although they are responsible for pediatric types, but these genes were not responsible for all childhood onset cases (Emily and Sara ,2021). For instance, the diseases caused by the genes CLN5, CLN6, CLN7, and CLN8 begin in late infancy. Until 2011, the CLN4 was not identified (Imler *et al.*, 2019). Most NCL genes encode proteins that function in the secretory and endo/lysosomal pathways. These are frequently lysosomal proteins, which are made up of transmembrane proteins (encoded by CLN3, CLN6, CLN7/MFSD8, and CLN12/ATP13A2) as well as soluble enzymes and proteins (encoded by CLN1/PPT1, CLN2/PPT1, CLN5, CLN10/MFSD8, and CLN13/CTSF). (Table 1.2). According to intracellular localization and

function NCL linked proteins (CLN1 to CLN14) might differ (Carcel *et al.*, 2015). In 2007 a mutation in *MFSD8* gene was reported first time in a patient of Turkish origin (Siintola *et al.*, 2007). Recently, 38 mutations are identified in *MFSD8* gene and most being homozygous missense mutations (Debnath *et al.*, 2020). More than 360 mutations that cause NCL, are included in the NCL Mutation Database (http://www.ucl.ac.uk/ncl/mutation) (Kousi *et al.*, 2012).

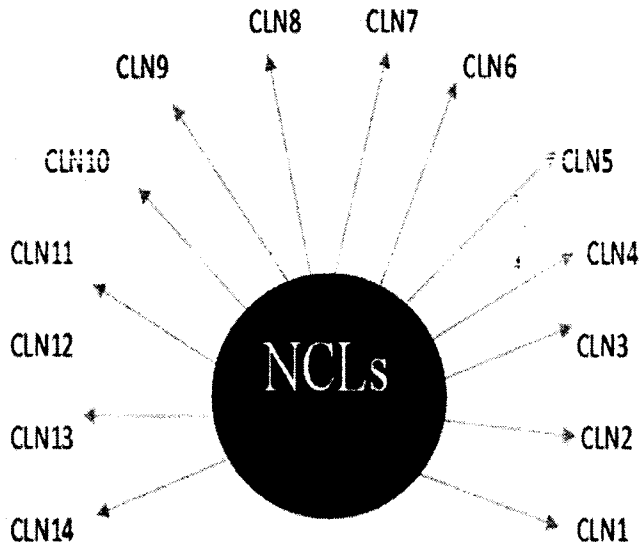


Figure 1.1 Neuronal Ceroid Lipofuscinosis are linked with fourteen different types and these were classified according to the age at disease onset (Parvin *et al.*, 2019).

1.6 Different Therapeutic Approaches for NCL Treatment

1.6.1 Enzyme Replacement Therapy

Enzyme replacement therapy is theoretically an easy and simple approach for treating an enzyme deficiency through a receptor -mediated processes. Furthermore, lysosomal enzymes may be released by cells and taken up by specific receptors in mannose-6-phosphate or mannose receptor systems. (Kornfeld, 1992). Recombinant enzyme that has undergone post-translational glycosylation and contains terminal mannose or mannsose-6-phosphate residues is endocytosed by cells after injection. Following intravenous injection, enzyme replacement therapy is reasonably effective in treating the systemic condition linked to a number of different lysosomal storage diseases (Solomon *et al.*, 2017).

1.6.2 Gene Therapy

Gene therapy strategies have shown encouraging results in animal models of different NCL. For instance, when compared to control mutant mice, newborn PPT1 mice treated with various intracranial injections of an AAV2 vector encoding human PPT1 displayed PPT1 enzyme activity near the injection sites, a decrease in storage material, a reduction in neurodegeneration, and progress in a number of behavioural tests, but the treated mice did not exhibit a decrease in seizure frequency or an increase in durability. (Kohlschutter *et al.*, 2019). Several cerebral injections of an AAV5-PPT1 vector were administered to young PPT1 ko mice, which decreased neurodegeneration and enhanced locomotor function while also prolonging the lifetime of the treated animals. (Sondhi *et al.*, 2012).

1.6.3 Cysteamine

For possible treatment of CLN1 disease the lysosomotropic drugs have same chemical activity to that of PPT1 as they have a target specific response to lysosomes and cleave thioester linkages similar to PPT1 (Zhang *et al.*, 2001).

1.6.4 Resveratrol

In cultured cells from CLN1 patients, resveratrol, an antioxidant molecule derived from the skin of red grapes, was shown to reduce oxidative stress and apoptotic markers.

ER stress and oxidative stress have both been associated to a number of LSDs, including INCL. (Wei *et al.*, 2008). In PPT1-deficient mice and fibroblasts, resveratrol treatment improved the energy profile and reduced apoptotic markers. (Wei *et al.*, 2011).

1.7 Aims and Objectives

The aim of this study was to identify the causative gene in Neuronal Ceroid Lipofuscinosis. The objectives of this study are as follows.

- Identification, clinical analysis of families affected with neurodevelopmental disorders from different region of Pakistan.
- Screening of these families for known genes of neurodevelopmental disorders by linkage analysis/DNA sequencing.
- Genetic analysis of unlinked neurodevelopmental disorders families for causative locus/gene.
- Genotype/Phenotype correlation and bioinformatics analysis for the pathogenic variant.

Table 1.1 Historical NCL classification of eight different CLN1-CLN8 diseases along with clinical phenotype (Dragos *et al.*, 2016)

Disease	Clinical Phenotype	Abbreviated Name	Eponym
CLN1	Infantile classic	INCL	Haltia-Santavuori
CLN2	Late- Infantile classic	KINCL	Jansky-Bielschosky
CLN3	Juvenile	JNCL	Batten-Spielmeyer-Sjogren
CLN4	Adult Autosomal Dominant	ANCL	Parry
CLN5	Late-Infantile classic	vLINCL	Finnish variant late infantile
CLN6	Early -juvenile/late - infantile	vLINCL	Lake-Cavanagh/Indian variant/Kufs
CLN7	Late-Infantile classic	vLINCL	Turkish Variant late infantile
CLN8	EPMR late-infantile variant	vLINCL	Northern epilepsy/EPMR

Table 1.2 Summary of the identification of genes that cause NCL with associated protein.

Gene	Associated protein	Localisation	Chromosome	References
CLN1	Plamitoyl-proteins thioesterase1(TPP1)	Lysosomal matrix	1p34.2	Jonathan *et al.*, 2019 Mole *et al.*,2015
CLN2	Tripeptidyl peptidase (TPP1)	Lysosomal matrix	11p15.4	David *et a.*, 2017 Emily *et al.*,2019
CLN3	CLN3	Golgi compartments/Lysosomal matrix	16p11.2	Favio *et al.*, 2019
CLN4	DNAJC5	Cytosolic, associated with vascular membrane	20q13.33	David *et al.*, 2017 Elliot *et al.*, 2019
CLN5	CLN5	Lysosomal matrix	13q22.3	Hannah *et al.*, 2017
CLN6	CLN6	ER-membrane	15q23	Davide *et al.*,2017
CLN7	MFSD8	Lysosomal membrane	4q28.2	Lisa *et al.*,2019
CLN8	CLN8	ER-membrane	8p23.3	Davide *et al.*,2017
CLN10	CTSD Cathepsin D	Lysosomal matrix	11p15.5	Elliot *et al.*,2019
CLN11	GRN Progranulin and granulins	Extracellular	17q21.31	Favio *et al.*,2019
CLN12	ATP13A2	Lysosomal membrane	1p36.13	Carcel *et al.*,2015 Dragos *et al.*,2016
CLN13	CTSF Cathepsin F	Lysosomal matrix	11q13.2	David *et al.*,2017 Meagan *et al.*, 2019
CLN14	KCTD7	Partially associated with plasma membrane	7q11.21	David *et al.*,2017 Favio *et al.*,2019

2. REVIEW OF LITERATURE

The most prevalent degenerative brain diseases in children are neuronal ceroid lipofuscinoses (NCLs), which are lysosomal storage disorders. One of the primary causes of permanent disability are neurodevelopmental disorders (NDDs), which affect approximately 7-14% of all children in developed countries and are one of the primary causes of permanent disability. (Vogel *et al.*, 2019). The disorder usually called as Batten disease is caused by a group of neurological disorder, the Neuronal Lipofuscinosis (NCLs), affecting every age and gender in a population; thus, globally distributed. (Meagan *et al.*, 2019). In spite of similar clinical findings, NCL- linked proteins have diverse cellular and sub-cellular localization. Numerous types of NCL have been reported according to mutations in NCL-linked proteins (CLN-CLN14) (Table 2.1). Fourteen types of NCL have been reported till date, with a detailed analysis of their genetics in NCL (Mahesh *et al.*, 2019).

2.1 Protein confined pathway in NCL

In NCL, the protein has been limited in different ways, as for example in lysosomes (CLN1 to CLN13) occur, in Endoplasmic Reticulum (CLN6 and CLN8), and in cytosol associated to vesicular membranes (CLN4 and CLN14) (Anil *et al.*, 2019). The most dominant types amongst 14 genetically categorized NCLs are type 1 and type 2 NCLs (Haltia and Goebe., 2013). NCL1 is distinguished from NCL 2 by the absence of palmitoyl protein thioesterase 1 (PPT1), while NCL type 2 is the outcome of deficiency of tripeptidyl peptidase 1 (TPP1) (Mole and Cotman, 2015).

2.2 Neuronal Ceroid Lipofuscinoses 1

Lack of palmitoyl protein thioesterase 1 (PPT 1) is a major cause of NCL1 (Davide *et al.*, 2017). The cytogenetics location of PPT1 is $1p^{34.2}$ (Hellsten *et al.*, 1993). CLN1 patients develop clinical sign and symptoms normally, however other symptoms usually appear after a year, including visual degeneration, anomalous involuntary movements (myoclonic and dystonic), fast paced conversation pattern, seizures and motor deterioration (Takano *et al.*,2008).

The lack of palmitoyl protein thioesterase 1 (PPT-1) enzyme, which work as lysosomal serine lipase with a standard α/β hydrolase and observed by electron microscope reveal granular osmiophilic (GRODs) on the rectal neuron (Vesa *et al.*, 1995).

2.3 Neuronal Ceroid Lipofuscinoses 2

CLN2 disorder, otherwise known as Neuronal Ceroid Lipofuscinosis type 2 is the result of autosomal recessive condition activated by variations in the *TPP1* gene. The mutation in the said gene (*TPP1*) lead to lack of activity of the lysosomal enzyme tripeptidyl peptidase 1(TPP1) (Emily *et al.*, 2019). CLN2 is the typical late infantile-onset arrangement. Patients of CLN2 have homologous genotype than CLN1 (Sohar *et al.*, 1999). Commonly CLN2 disorder present among the children from 1 to 4 years in which change is consistent till psychomotor reversion starts in the second year of life, observed via seizure and epilepsy (Nickel *et al.*, 2018). The essential gene located at 11p15 (*CLN2*) translates for tripeptidyl peptidase 1(TPP1) (Bessa *et al.*, 2008). CLN2 can be investigated by extent of the TPP1 enzyme activity in the lysosomes, compound heterozygote or homozygote change in CLN2 (Romina *et al.*, 2013). CLN2 disease is caused by abnormal function of lysosomal enzyme known as tripeptidyl peptidase 1 (TPP1) (Zoltan *et al.*, 2017).

The lack of the lysosomal enzyme tripeptidyl peptidiase-1 is caused by variation in *CLN2* gene (Whiting *et al.*, 2014). It is autosomal recessive inheritance trait; thus 14 different genes express the different types of NCL diseases (Table 2.1) (Katz *et al.*,2017). *TPP1* gene encodes lysosomes enzyme tripeptidyl peptidase 1 which is linked with serine carboxy proteinase family. It is an aminopeptidase that is important in the processing of neuron-specific trophic factors and releases N-terminal tripeptides from a polypeptide. (Ezaki *et al.*, 2000). Moreover, a small portion of this protein is observed in human endopeptidase activity., *TPP1* mutations result in late infantile neuronal ceroid lipofuscinosis (LINCL), which affects children between the ages of 2 and 4 and is characterised by seizures, ataxia, and progressive cognitive and visual impairment. (Wei *et al.*, 2019).

It was predicted that pathogenic CLN2 mutations frequently cause the typical late infantile phenotype (LINCL). The *TPP1* lacking individuals generally exhibit one of the three phenotypes conferring to their age at inception, late infantile (LI), variant juvenile (vJ), or variant infantile (vI) (Williams *et al.*, 2006). In other cases, the phenotype of individuals be

like juvenile forms (J-NCL) and the patients were consistently compound heterozygotes (Mole *et al.*, 20015). A rare infantile phenotype, CLN2 patients has also been studied which determines, onset of the first year of age (Ju *et al.*, 2002). On the past 20 years 30 cases of NCL disorders from Argentina have been reported (Kohan *et al.*, 2013).

The vJ-NCL phenotype includes an extended development and late onset age (4.5 – 10 years of age), and the rare infantile form features onset at almost 1 year of age. The LI-NCL phenotype is the most prevalent CLN2 form worldwide, characterised by early-onset seizures (2 – 4.5 years onset age), developing encephalopathy, and visual failure. (Ju *et al.*, 2002).

2.3.1 Prevalence of *TPP1*

Data from paediatrics population demonstrate a prevalence of approximately 2 to 4 per 100,000 live births due to the neuronal ceroid lipofuscinoses which is amongst the common neurodegenerative conditions (Mink *et al.*, 2013). Topographical evidence is known for 356 people (92% of cases), the majority of whom (217) are from Europe. Other patients include those from North America (78), South America (28), Asia (9), the Middle East (9), a mix of Europe and other nations (8), Central America (5), and Africa (2) (Figure 2.1) (Emily *et al.*, 2019).

2.3.2 Clinical Characterizations *TPP1*

NCLs are clinically classified by epileptic seizures, visual impairment, psychomotor degeneration and premature death, moreover, inherited in autosomal recessive way. (Anastasiya *et al.*, 2020). Disease commonly expresses itself at about three years of age with decline motor growth and seizures (Kohlschutter *et al.*, 2016). The TPP1 in human beings is consistently distributed in some organs and tissues, generally those linked with the production of neuropeptides and peptide hormones (Adam *et al.*, 2004). The TPP1 is generally an aminopeptidase that release N-terminal tri-peptides from polypeptide and fundamentally elaborate in neuron specific trophic aspects. The gene *TPP1 (NM 000391.3),* which encodes the lysosomal exopeptidase, corresponds to chromosome $11p^{15}$. One missense variant (p. Cys45Arg) was found in exon 2 of the *PPT1* gene, two missense variations (p. Pro238Leu and p. Val236Gly) were found in exon 7, and one frameshift variation (p. Glu178Asnfs)

was found in exon 5 of the gene in 44% of the patients, the prevalent variation p. Pro238Leu was found. (Gardner *et al.*, 2021). Mutations in the CLN2 gene, which encodes the lysosomal serine protease tripeptides peptidase 1, result in CLN2 disease. (Sleat *et al.*, 1997). In the whitest populations, more than half of patients with CLN2 disease have been known to have two mutations (c.5091G-A [splicing mistake] and c.622C-T [nonsense mutation]), which are caused by failure of tripeptidyl peptidase 1 activity. (Kwi-Hye *et al.*, 2008).

2.3.3 Classification of *TPP1* gene

There are four significant scientific subtypes identified on the basis of age which include infantile, late infantile, juvenile and adult types (Karen *et al.*, 2019). Failure in TPP1 compound movement effects intracellular progress of protein rich material called ceroid lipofuscin, which is linked with glial stimulation and neuronal misfortune (Ina *et al.*, 2019). The new course of action is coordinated along seven demonstrative factors: (1) influenced gene; (2) mutation; (3) biochemical phenotype; (4) clinical cumulative /phenotype; (5) ultrastructure highlights; (6) level of useful debilitation; and different comments (extra hereditary, ecological, or logical highlights; (Williams *et al.*, 2012). CLN2 disorder is caused due to the aggregation of lysosomal stockpiling problems (LSD) caused by changes in the TTP1 quality that cause an insufficiency or complete lack of the solvent lysosomal chemical tripeptidyl peptidase-1 (Matthew *et al.*, 2019).

Late-infantile neuronal ceroid Lipofuscinosis is also known as lysosomal storage disease which is one of the most frequently dominant family of Batten disease (Sleat *et al.*, 2016). Worldwide, the prevalence of NCL has been reported 1:1,000,000 to 1:14,000 and documented as one of the most occurring childhood onsets neurodegenerative disorders (Haltia *et al.*, 2013). The disorder is linked to heterogeneous group of NCL which leads to, impairment in vision epilepsy, dementia and other normal skills is young individuals (Dragos *et al.*, 2016). In 2018, 140 disorder producing mutations in *TPP1* gene have been found to be inked with *CLN2* in which frequently occurring mutations are c.509-1G>C and p. Arg208Ter (Jayesh *et al.*, 2019).

2.3.4 Biological significance of *TPP1* gene

During the post-acidification, process the inactive proenzyme form is treated to a 46

KD a protein. The in active 66-KDa precursor is released by CLN2 protein which is catalytically changed (Lim *et al.*, 2001). From the amino acid terminal of small polypeptides, TPP1 cleave tripeptides (MW<15,000) at pH 4-4.5 (Robert *et al.*, 2002), TPP1 is a lysosomal protease that needs acidic pH for its activity. Small proteins causing CLN2 disease are prevented from developing by the inactivation of the aminopeptidase activity of TPP1. (Anil *et al.*, 2019). The CLN2 quality is commonly passed on and formatively directed. TPP1 is gradually articulated in the human brain starting at the age of two. (Kurachi *et al.*, 2001). The identified TPP1 mutations cover the entire protein structure and may be related to the effectiveness of the enzyme's function. Failure of neuropeptide deficiency and significant assembly of ATP synthase subunit "c" are caused by the absence of TPP1 activity. (Pamer *et al.*, 2013). Gathering of subunit "c" has been identified in different arrangements of NCL and other lysosomal storage disorder, indicating that this could not be the fundamental metabolic error in TPP1 absence (Ryazantsev *et al.*, 2017). Lysosomal storage abnormalities are known to be caused by a variety of mutual pathogenic cascades, including altered lipid trafficking autophagy, altered calcium homeostasis, and oxidative stress (Vitner *et al.*, 2010)

2.3.5 Diagnostic and Clinical Relevancy

Investigation of neuronal ceroid lipofuscinosis disorder may be prolonged by a mixture of clinical results, TPP1 enzyme insufficiency, or molecular conclusions in TPP1 (Fietz *et al.*, 2016). Studies of NCL subtypes, slightly rely on histopathological methods, such as an electron microscope observation of auto fluorescent storage material pathology, managed by an experiment of auto fluorescent storage material morphology, managed by and experimental observation of disease initiation and symptoms (Mole *et al.*, 2005). Examination of white blood cells TPP1 activity supports diagnosis for TPP1 linked diseases (Fietz *et al.*, 2016). However, this suggests a direct examination for CLN2 disease which can lead to significant findings on disease development and neurodegeneration (Nickel *et al.*, 2018). In comparison to biochemical analysis, hereditary analysis could be utilized to test several aetiologies, and possibly lead to a patient's phenotype. This suggested that no specific doubt of an ethology is necessary, next generation sequencing (NGS) based tests as a tool for prior to analysis of hereditary disorders are sufficient NGS techniques, for instance, whole sequencing (WES) has proven to be of late helpful tools for improving NCL subtype arrangement, specifically when transformation in several qualities cause equivalent covering collections (Patino *et al.*, 2014).

2.4 Neuronal Ceroid Lipofuscinoses 3

The common observed subtype of NCL due to the recessive loss of activity alters in CLN3 (ceroid lipofuscinosis neuronal protein 3) (Kitzmuller *et al.*, 20018). This is the most common mutation observed globally with intragenic removal but not entirely eliminating the function and this translates a preserved 438-amino acid poly topic membrane product most possibly in Golgi compartments, synaptic vesicles and lysosomes/ endosomes (Sabeteeshan *et al.*, 2018).

2.5 Neuronal Ceroid Lipofuscinoses 4

The amino acid substitution or single amino acid removal L116Δ in the SV protein CSPα, which is set by the human *DNAJC5* gene, is the cause for autosomal dominantly inherited NCL *CLN4*. This change is observed to manifest in an individual between 25 and 46 years of the age (Elliot *et al.*, 2019).

2.6 Neuronal Ceroid Lipofuscinoses 5

CLN5 is an N-glycosylated protein with 407 amino acids produced by lysosomes and has a vital importance as sensor in lysosomal trafficking. (Parvin *et al.*, 2019). CLN5 alter an ER membrane protein which suggests its functions comprising of endocytosis of lysosomal protein and specific carriage of lipids and proteins crucial for the role and acidification of the lysosome. It is a soluble lysosomal protein with elective roles covering neuronal care, neurogenesis, synaptic endocytosis and autophagy. (Hannah *et al.*, 2017). *CLN5* is the commonly described membrane protein which is present in endoplasmic reticulum, generally associated with common sign and symptoms including seizures, cerebral decline, gait problems and retinopathy, with patients first identified clinically between 2 and 8 years of age (Williams *et al.*, 20016).

2.7 Neuronal Ceroid Lipofuscinoses 6

A subdivision of the patients with described (*CLN6*) mutations are observed in adults NCL or Kufs disease (type A). In majority of cases, teenage symptoms include variable form of seizures, decrease in cognitive potential and ataxia (Chigure *et al.*, 2019). CLN6 patient are a progressive form of late infantile-onset NCL (Masayuki *et al.*, 2017).

The clinical appearances include visual decline, dysarthria, motor evolving delay, ataxia, seizures. The gene is located at 15q21-23 and responsible for encoding a transmembrane protein of endoplasmic reticulum of unidentified purpose (Mole *et al.*, 2015).

Table 2.1 NCL Classification with Protein size and Cellular Localization

Gene	Classification and protein Size	Encoded Protein	Sub cellular localization
CLN1	Soluble protein, 306 aa	PPT1	Lysosomal lumen, Extra lysosomal vesicles, areas in neurons
CLN2	Soluble protein, 563 aa	TPP1	Lysosomal lumen
CLN3	6 TM protein, 438 aa	CLN3	Late endosomal/ lysosomal membrane
CLN4	Soluble protein, 198 aa	DNAJC5. (CTSF)	Cytosolic, vesicular Membranes
CLN5	Soluble protein, 407 aa	-	Lysosomal lumen
CLN6	7 TM protein, 311 aa	CLN6-protein	ER-membrane
CLN7	12 TM protein, 518 aa	MFSD8	Lysosomal membrane
CLN8	5 TM protein,286 aa	CLN8	ER-membrane
CLN10	Soluble protein, 462 aa	(CTSD)	Lysosomal lumen
CLN11	Soluble protein, 593 aa	Progranulin and granulins	Extracellular
CLN12	10 TM protein, 1180 aa	ATPase 13A2, KRPPD	Lysosomal membrane
CLN13	Soluble protein, 484 aa	CTSF)	Lysosomal lumen
CLN14	Soluble protein, 289 aa	KCTD7)	Cytosolic, partially associated to membranes

* (Anil B *et al.*, 2019)

*PPT1 Palmitoyl protein thioesterase 1, *CSPα Cysteine-string protein alpha, TTP1 Tripeptidyl peptidase 1 and *CTSF Cathepsin F, *KCTD7 Potassium channel tetramerization domain containing protein 7

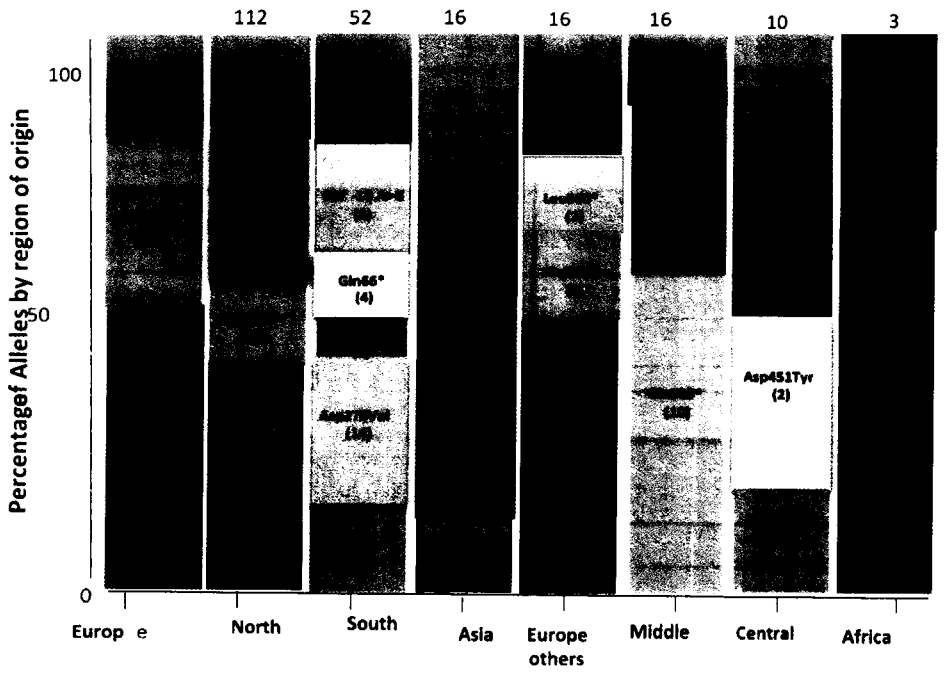


Figure 2.1 Most common alleles listed in the TPP1 locus-specific database by region of origin. The number of times an allele was encountered is shown in parentheses. North America includes New found land (Emily *et al.*, 2019).

The *CLN6* gene that induce mutation in a patient is recessively inherited neurodegenerative disorder that is present on chromosome 15q21-23 (Hanlin *et al.*, 2002). CLN6 mainly contain seven expected transmembrane domains with 311-amino acid protein (Wheeler *et al.*, 2002). CLN6 has been explored in lysosomal acidification as a transmembrane protein (Joseph *et al.*, 2019). Usually, the individual is affected by a usually small brain weighing 15-50% of the likely weight for the gestational age (Rakheja and Bennett, 2018). For NCLs activated by absence of soluble lysosomal enzyme, enzyme substitution therapy, small cell therapy, gene therapy approaches are used to treat the patients. (Wong *et al.*, 2010). In 2017 the enzyme substitution therapy, The European Union and the United States' food and drug administrations have both approved recombinant human tripeptidyl peptidase-1 cerliponase alpha for use in CLN2 patients of all ages (Markham and Cerliponase, 2010). In CLN6, there are 70 disorder causing mutations are known which involve loss of CLN6 protein (Jacob *et al.*, 2019). The cluster of autosomal recessive neurodegenerative disorder which is described by the gathering of auto fluorescent lip pigment in different tissues are known as neuronal ceroid lipofuscinosis (Alessandro *et al.*, 2002). Endoplasmic reticulum membrane protein has been determined by functions such as selective transport of lipids, endocytosis of lysosomal proteins and proteins essential for the role and acidification of the lysosome (Hannah *et al.*, 2017).

2.7.1 Occurrence and Forms of CLN6 disease

Collectively, it is the most common cause of neurodegenerative disorder in children, through an occurrence of 2-4/100,000 live births (Jacob *et al.*, 2019). CLN6 disorder is present in two forms, variant infantile (vLINCL), and second is adult-onset NCL (also called as Kufs disease) (Natalia, *et al.*, 2009). Along with the first form the age of affected individual is between 18 months to six years and death commonly appear by age 12-15 (Canafoglia *et al.*, 2015).

2.7.2 Symptoms of CLN6

The most generally associated sign and symptoms include cognitive decline, seizure, gait problems and retinopathy with patients basic reporting to the clinic between 2 and 8 years of age (Wheeler *et al.*, 2002).

2.7.3 Classification

The biological classification of NCLs is well established and sufficiently explains pathophysiologic origin of the disease process. (Table 2.2) (Jalanko and Brauke, 2009). CLN6 disease could appear as late infantile or adult onset where adult-onset type is linked with kufs disease (Table 2.2) which has two subtypes: childhood and teenage kufs disease (Berkovic *et al.*, 2019). Kufs disease has been sub-classified into two types (Masayuki and Ayako, 2017). Type A expresses symptoms such as triggers through advanced myoclonic epilepsy, with later progress of dementia and ataxia and unfortunately has no visible symptoms. Type B is characterised by dementia with cerebral and/or extrapyramidal motor signs (Mole *et al.*, 2005). NCL and kufs disease is prevalent however not given to attention it warrants (Sharp *et al.*, 2003). It is about 40% of variations of late-childish NCL cases with *CLN6* mutations, both alleles have conversion anticipating protein truncation or an intensively common protein (Moore *et al.*, 2008).

These alterations, however, were only observed in two kufs cases and were present on a single allele, confirming the possibility that persons with kufs disease may still have important CLN6 activity. The development of knowledge regarding NCL6's capacity will help determine whether the changes in kufs disease are "milder" or whether the phenotypic differences are anticipated to change genes (Todor *et al.*, 2011). As a distinctive feature if CLN6 disease, certain reports show changes in metal homeostasis pathways by frequent accumulation of zinc and manganese and common cell signalling associated to AKT/GSK3 and ERK/ MAPK pathway. Changes in neurite formation in CLN6 disease have been correlated with reduced reliability and cause of the CLN6 interactor, failing response mediator protein 2 (CRMP-2), which may contribute to neuronal dysfunction and encephalopathy (Benedict *et al.*, 2009). It has seven transmembrane domains, a luminal C terminus, and a cytoplasmic N terminus. (Heine *et al.*, 2007). According to Lyly et al. (2009), CLN6 can bind to the NCL protein CLN5 and the subsiding response transitional protein-2 (CRMP-2). Cells from patients with CLN6 disease may have an elevated level of lysosomal pH (Benedict *et al.*, 2009). While lysosomal breakdown of an endocytosed protein was reduced, at least some lysosomal enzymes appear unaffected. (2004) Heine et al. A study of definite changes associated with a variant late-infantile NCL in vitro indicates that the aberrant protein is degraded more quickly. (Kurze *et al.*, 2010).

The pathogenic variant known as a truncation mutation is projected to cause protein truncation and total loss of CLN6 function in the afflicted allele. (Figure2.2). Special other mutation' refers to missense mutations at positions Arg103, Arg136, Met241 and Asp256, which when combined with another missense change are associated with adult disease onset and when combined with a truncation mutation are associated with late infantile-onset with visual impairment. Other mutation position to variants that affect amino acid than the 4 listed above and keep *CLN6* role to varying degrees; in kufs disease no these are present on exon 6 (Berkovic *et al.*, 2019). Truncation mutations may result in CLN6 protein that is severely deficient or absent, which is consistent with the earlier recommendation that the severity of the mutation and the age at disease onset are correlated (Kousi *et al.*, 2012). Only 15/42 (36%) late infantile onset individuals and none of the adult patients exhibited two truncation alterations (Berkovic *et al.*, 2018).

2.8 Neuronal Ceroid Lipofuscinoses 7

zzCLN7/MFSD8 gene has 35 different identified mutations. These mutations in gene are the reason of collectively contribute in the manifestation of the NCL7 disease (MIM #610951). (David *et al.*, 2017). The first case of CLN7 has been reported in Turkish family hence the name Turkish NCL which is also linked with the variant of late infantile onset (Eija *et al.*, 2007). These altered late infantile onset types are genetically heterogeneous (Mancini *et al.*, 2015). The location of gene is at chromosome 4q28.1-28.2, and basic sign and symptoms are myoclonus, measured by motor decline, seizure, visual impairment, and dementia which could be a juvenile-onset (Maria *et al.*, 2009).

2.9 Neuronal Ceroid Lipofuscinoses 8

The *CLN8* is associated with an endoplasmic reticulum protein (Tsankova *et al.*, 2007). Cathepsin D has significant part in autophagy-lysosomal system, and it generates a neuropathological disorder that be similar in characteristics to that of NCL10 phenotype (Chigure *et al.*, 2019). The age of onset is typically five to ten years and patients mainly present with pre- exiting seizure and related symptoms are found advancing into a decline in motor and cognitive abilities and visual loss. The gene is present at 8p32. The *CLN8* mutation causes variation in transmembrane protein of the endoplasmic reticulum which may work as endoplasmic reticulum-Golgi intermediate complex, but its actual purpose is unidentified (Geraets *et al.*, 2016).

2.10 Neuronal Ceroid Lipofuscinoses 9

CLN9-lacking cells have an apparent increased apoptosis but enhanced growth combined with very low ceramide levels (Angela *et al.*, 2006).

2.11 Neuronal Ceroid Lipofuscinoses 10

CLN10 a primary onset associated *NCL*, is an inherited disorder its most prominent feature includes microcephaly because of brain atrophy, lack of neonatal reflexes and respiratory deficiency. The chromosomal location of the gene corresponds to 11p15.5 (*CLN10/CTSD*) (Steinfeld *et al.*, 2006). activity of the lysosomal enzyme of the aspartic protease cathepsin D. Neuronal Ceroid Lipofuscinoses 11 Neuronal Ceroid lipofuscinosis 11 disorders can be distinguished due to homozygous change in the progranulin quality (GRN) (Vincent *et al.*, 2020). ATP13A2 variant is associated with CLN12 illness which is called Kufor-Rakeb disorder (Isabelle *et al.*, 2020).

2.12 Neuronal Ceroid Lipofuscinoses 11

Neuronal Ceroid lipofuscinosis 11 disorders can be distinguished due to homozygous change in the progranulin quality (GRN) (Vincent *et al.*, 2020) ATP13A2 variant is associated with CLN12 illness which is otherwise called Kufor-Rakeb disorder (Isabelle *et al.*, 2020).

2.13 Neuronal Ceroid Lipofuscinoses 12

The well-known aetiology of Kufor-Rakeb syndrome is mutations in the ATP13A2 (CLN12) gene, a rare form of autosomal recessive infantile or early-onset, levodopa-responsive Parkinsonism with dementia. Patients with Kufor-Rakeb syndrome exhibit extrapyramidal involvement in addition to the normal NCL symptoms. The CLN12 (ATP13A2) gene encodes a lysosomal transmembrane protein of 36 kDa with 10 predicted transmembrane domains, commonly known as KRPPD, PARK9, HSA9947, or RP-37C10.4. (Carcel *et al.*,2015).

2.14 Neuronal Ceroid Lipofuscinoses 13

The CLN13 gene, which encodes cathepsin F (CTSF), was firstly reported to cause neurological disease in mice by accumulating autofluorescent material in neurons of the cerebral cortex, cerebral Purkinje cells, hypothalamus, and other parts of the brain. (Tang *et al.*, 2006). Neurological inflictions begin to appears between 12-16 months of age and can be identified by features like lack of synchronization, weakness in muscles, early death (Anil *et al.*, 2019).

2.15 Neuronal Ceroid Lipofuscinoses 14

Initial-onset progressive myoclonic epilepsy, with seizures, which is followed by myoclonic movement and visual loss has been reported in patients with CLN14. It is known to cause changes in the role of potassium channel tetramerization domain surrounding protein 7 (*KCTD7 gene*), that is present in the cytoplasm and parenthetically associated with cellular membrane on Cytogenetic location $7q^{11.21}$ (Staropoli *et al.*, 2012). CLN2 disease receives attention as enzyme substitution therapy with cause of biosynthetically produced TPP1 into a cerebral ventricle (Schulz *et al.*, 2018). The trial finding that the manose-6-phosphate pathway deals with a helping of the CLN5 gene point in any case suggests a course of treatment. (Isabelle *et al.*, 2019). It suggests that cross-adjustment-based corrective techniques, such as complicated replacement therapy, quality treatment, and early microbe transplanting, are potentially effective to try in the near future. (Sleat *et al.*, 2015). In 2017 the enzyme replacement therapy by a recombinant human tripetidyl peptidase-1 cerliponase alpha has been recognised for CLN2 patients with approval form the Food and Drug Administration, as well as for CLN2 patients in the European groups at various ages. (Markham *et al.*, 2017). Other therapies right now are to controller symbols and expressions, for instance, seizures and mental issues. However, NCLs are examined to be confirmed, a variations of treatment methods have been confirmed in an animal model, around of them with a hopeful therapeutic result.

The active treatment for NCL forms were initiated by impairment of soluble lysosomal enzymes in the substitution of the imperfect enzyme is through infusion of the recombinant protein, cell-based tactic and virus mediated gene transfer. In 2019 the recent report indicating the effectiveness of an enzyme substitution therapy to lower the speed of disease development in patients with CLN2 disease. The progress in active therapies for

NCLs caused by the mutations in transmembrane proteins, in evaluation, is further stimulating possible treatment probabilities include expansion approaches, neuroprotection, or small-molecule therapies (Alfried *et al.,* 2019). This study is designed to organise common gene variants in Pakistani consanguineous families identified with NCL.

Table 2.2 Classification of NCL6 based on the age at the onset of clinical manifestations

CNCL	CLN10
INCL	CLN1, CLN14
LINCL	CLN2, CLN1, CLN5, CLN6, CLN8, CLN10
JNCL	CLN3, CLN1, CLN2, CLN8, CLN CLN9
ANCL	CLN4, CLN6, CLN1, CLN10, CL CLN13, CLN11

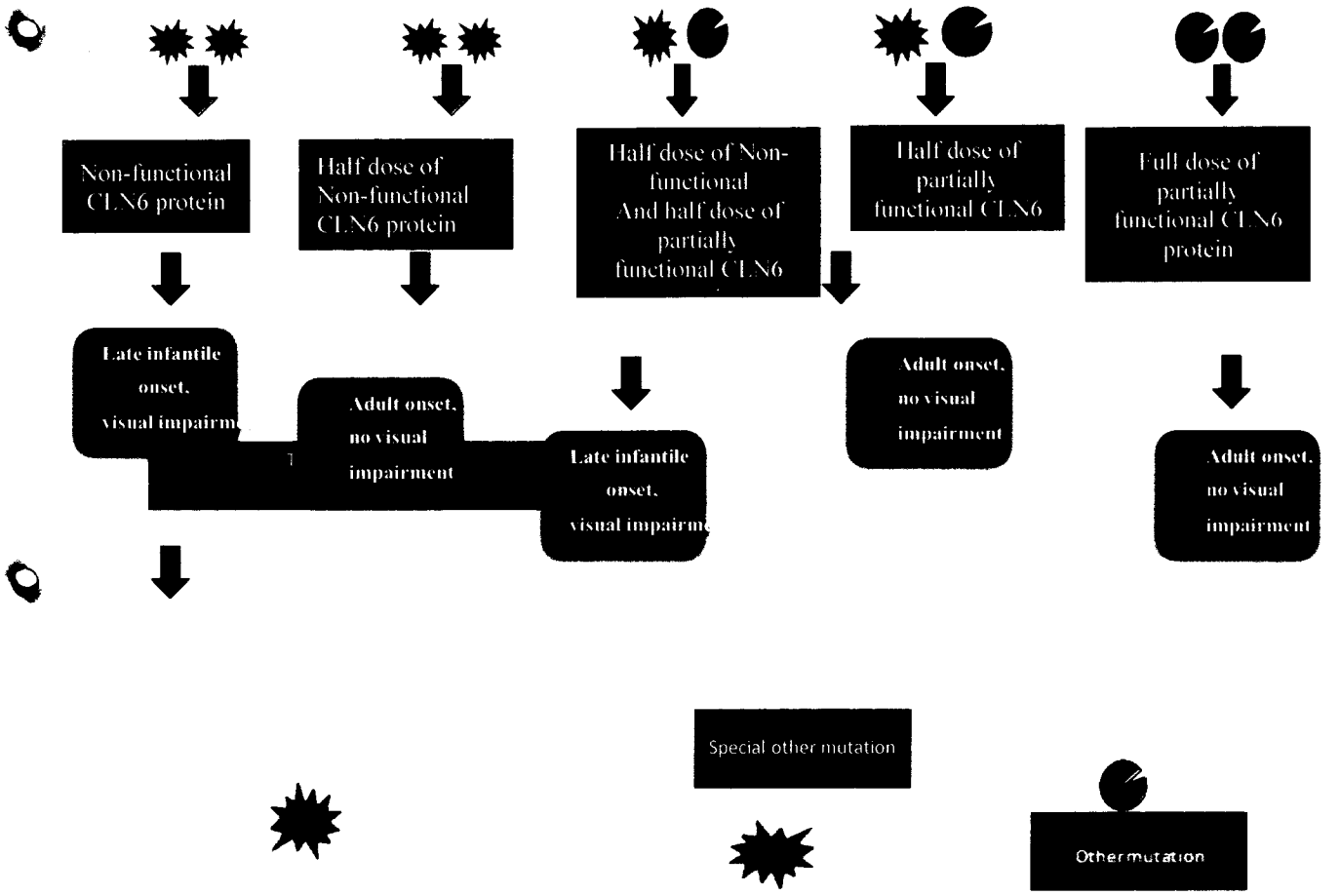
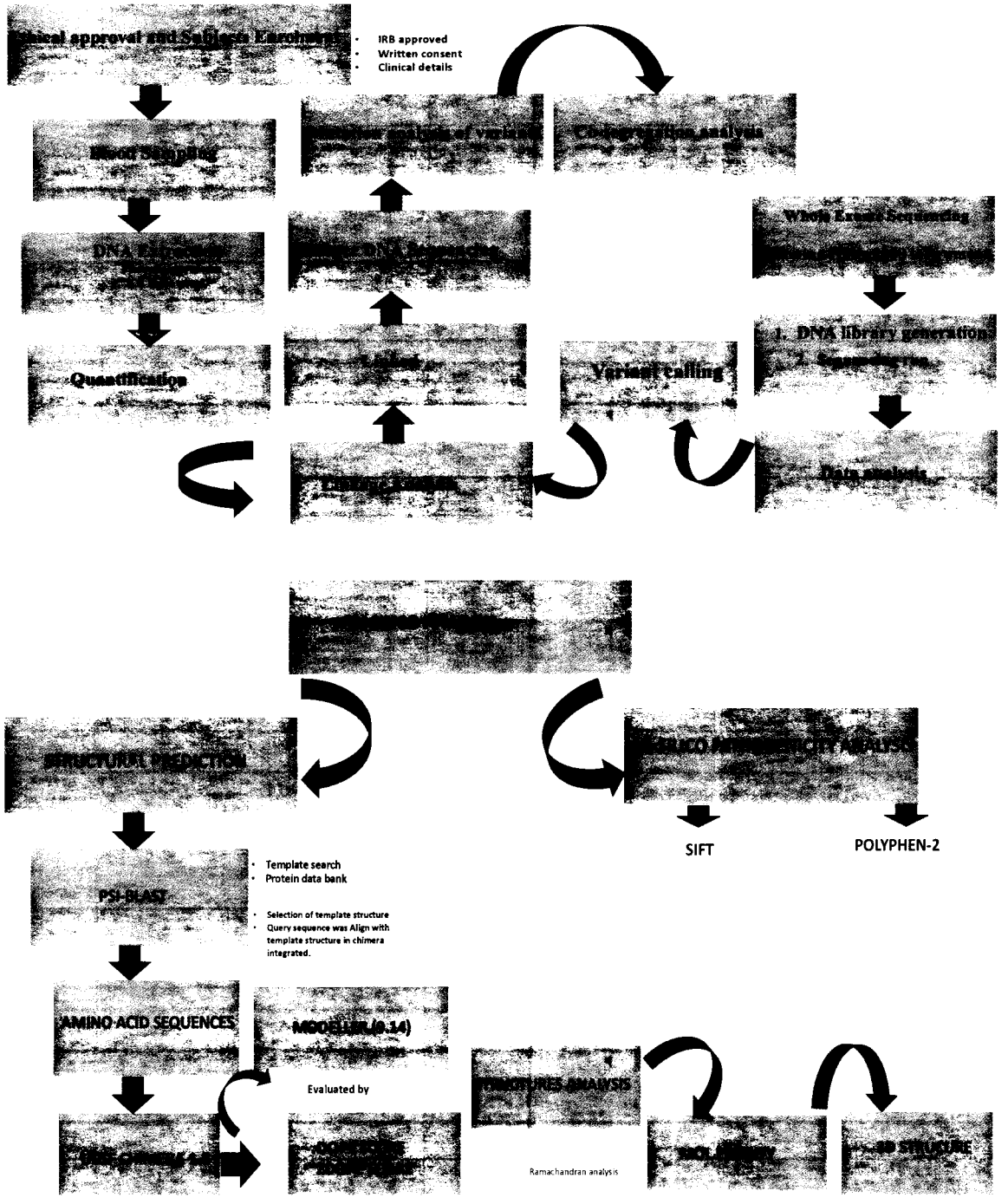


Figure 2.2 Schematic representations of genotype-phenotype correlations in CLN6-associated diseases. 'Truncation mutation' refers to a pathogenic variant predicted to result in protein truncation and complete loss of function of CLN6 from the affected allele. (Berkovic *et al.*, 2018).

3. MATERIALS AND METHODS

A brief road map of methodology used for the genetic analysis is described below (Figure 3.1)



3.1 Participants and Ethical Consent

The research was approved by Institutional Review Boards (IRB) of International Islamic University Islamabad and National Bioethics Committee, Islamabad, Pakistan. All families (A to K) belong to different areas and with variety of ethnic groups of Pakistan. The relevant medical background from the families was collected carefully, and the initial diagnosis was confirmed by a neurologist.

3.2 Families Enrolment

Families having affected individuals of neuronal ceroid lipofuscinosis were identified for selection from different areas of Pakistan. Clinical history of diseased individuals was taken and determined personally after visiting the affected families. Moreover, mode of inheritance was determined by conducting interviews with various family members were conducted to ascertain the pattern of inheritance. Pedigrees were constructed using the Cyrillic® software (Cyrillic for Windows 3.1) and Macromedia® FreeHand® software. After enrolling families for the study, laboratory work started.

3.3 Identification of Affected Individual and Collection of Samples

Clinical information about the patients was obtained with the use of medical records. Using peripheral blood samples; genomic DNA was extracted, following the standard protocol of phenol chloroform protocol.

3.4 DNA Extraction Procedure

All blood cells were liquefied for the breakdown of red blood cells. Washing buffer also known as TE buffer was mixed in each sample by raising the volume in the 50ml falcon tubes up to 40ml. At 2700 rmp the blood samples were centrifuged for 20 minutes in Beckman TJ-6 centrifuge 20°C temperature. The supernatant was discarded about 10-15 ml and remained pellet was broken by soft tapping. Shake the sample tubes strongly by adding the washing buffer again. At 3000 rpm at 20°c to 25°c temperature the sample tubes were

centrifuged. Until all RBCs were removed these two steps were repeated. For the digestion of protein added the mixture of 6 ml of Tris.NaCl and EDTA/A1 buffer, 30µl of Proteinase K (with the concentratin10 ug/µl) and 150 µl of 10% SDS (each for 10ml of blood volume). To separate the protein, sodium chloride was used which binds with DNA, making it dissolve in sodium. The samples were placed in shaking incubator for overnight incubation at a 37 ^{o}C and speed of shaker was set at ~250 rpm. 0.1ml of 6 molar sodium chloride (NaCl) per 1ml of blood volume was added for proteins for its precipitation by vigorously mixing the sample for 15 minutes and later placed the sample on ice. At ~2700 rpm the samples were centrifuged for 15 minutes at 20 ^{o}C.

Two layers were formed into tube, the lower layer was discarded and the upper layer taken out in another falcon tube. By using volume of Isopropanol equal to separated supernatant the precipitation of DNA was done. Some white threads of DNA were appeared after the slight inverting of falcon tubes. After that tubes with DNA samples were again centrifuged at 2000rmp for 10 minutes. DNA appeared as pellet in the bottom of tube after centrifugation, Isopropanol was discarded and 70 % ethanol was added for washing of the DNA pellet. To preserve DNA, the DNA pellet was dissolved in 1.5 ml of autoclaved Tris-EDTA DNA buffer. To ensure appropriate DNA mixing in the TE buffer. The samples were placed in a shaking incubator overnight at a temperature of 37 °C to ensure that the DNA in the TE buffer is properly mixed. To inactivate any remaining nucleases, the water bath heat shock was given to dissolved DNA at 70 C for about 50 minutes. All the samples were carefully sealed, and the DNA was then frozen for later use.

3.5 Spectrophotometry

To check the quality of the isolated DNA, spectrophotometry was performed. The precise wavelength was set to 280nm, 230 nm, 260nm, and 320nm and the absorbance ratios A260/A280 and A260/A230 was resolute aimed at finding the impurities in DNA through the abstraction procedure.

3.6 Check PCR products on Agarose Gel

To prepare the 2% Agarose gel 2 grams of agarose powder was mixed in 100mL of TBE 1x buffer.

Agarose powder and TBE 1x buffer was heated in a microwave for 2 minutes to mix them well.6ul of Gel Red (equivalent of ethidium bromide) was added and poured into the gel casting tray. The combs were placed and allowed to set for 30 minutes. 3ul of loading dye was mixed with 7ul gel and gel and was run at 100 V for 30 minutes.

3.7 Primer Designing

We used Ensembl Genome Browser to get sequence and design the primers of genes. (http://www.ensembl.org/Homo_sapiens/Info/Index?db=core). Primer3 software 0.4.0 was used to design the primers. The method by which primers were designed, is given below: The length of primers was in-between 20 to 24 base pairs. For optimum polymerase chain reaction efficiency, the temperature difference between forward and reverse primer were kept on temperature between 59 to 62^0c. In each primer the guanine – cytosine (GC) base content was kept between 40-60%. For the prevention of secondary structure and primer dimmers formation during the reaction, primers were designed in such way that there was no inter or intra-primer efficiency extending for more than 3 bases. The specific and precise primer sequences were selected with 100% accuracy to the amplified region. For the accuracy and verification of the selected primer sequence In-silico scrutiny were done using the UCSC Genome browser. Each primer was designed according to Families A-K, along with *CLN5, CLN6, TPP1, MFSD8* genes, as shown in the tables. (Tables 3.1,3.2 3.3 ,3.4,3.5 and provide more information (Table 3.6).

3.8 Linkage analysis

For identification of segregation of disease with the genome under study, Linkage analysis is a prevailing tool. The data was arranged in Microsoft Excel Sheet and Easy Linkage package and was used for statistical analysis for confirmation of the linkage.

3.9 Exome Sequencing

The whole exome sequencing (WES) was done in probands (Mencacci *et al.*, 2016), in Macrogen, Korea. To generate barcoded whole-exome sequencing libraries the target enrichment was performed with 2μg genomic DNA using the SureSelect XT Human All

Exon Kit version 6 (Agilent Technologies, Santa Clara, CA, USA). On the HiSeqX platform (Illumina, San Diego, CA, USA), libraries were sequenced with 50 coverage. To quality assessment of the sequence reads was achieved by generating QC statistics with Fast QC (http://www. bioinformatics.bbsrc.ac.uk/projects/fast qc).

Only exonic and donor/acceptor splicing variants were screened for in the bioinformatics classification techniques. Substantial significance was assigned to rare variations (0.01% in public datasets, including the 1000 Genomes project,Exome Aggregation Consortium [ExAC v0.2], NHLBI Exome Variant Server, whole Genomics 69, and variations in genes previously connected to neuronal ceroid lipofuscinoses and other neurological diseases that were relevant to recessive (homozygous or compound heterozygous) or a de novo model.

Table 3.1 Primer sequencies used for Co-segregation analysis of *CLN5* gene

Family ID	Primer	Sequence	Temp	Product Length
A	Forward primer	CCCATGGTTGTGTCACAGTT	65.3 ^{0}C	441bp
A	Reverse primer	CCAGTTCCCCACAAACCTATTC	63.5 ^{0}C	
B	Forward primer	TGATGTTACCACCGCACTCT	63.2 ^{0}C	521bp
B	Reverse primer	CTAACTCGCTCACTTTGGCC	63.2 ^{0}C	
C	Forward primer	GCATGGAAGAACAGCTGAACA	65.3 ^{0}C	570bp
C	Reverse primer	CCAGTTCCCCACAAACCTATTC	65.3 ^{0}C	

Table 3.2 Primer sequences used for Co-segregation analysis of *TPP1* gene

Family ID	Primer	Sequence	Temp	Product Length
D	Forward primer	CAGGCCCCAAATCTCTTGTG	64.6^{0}C	597bp
D	Reverse primer	CTGACCACCCAGTAGCCATC	66.7^{0}C	
E	Forward primer	CCTGGTTGCTGTACTCCTGT	61.7^{0}C	624bp
E	Reverse primer	TAGCCATCAGAAAGTGCAGC	61.8^{0}C	
F	Forward primer	GGTGGGGTGAGTTGTAAGGT	62.7^{0}C	
F	Reverse primer	TGAGCCAGGTCTGAGTCATG	64.3 ^{0}C	624bp

Table 3.3 Primer sequences used for Co-segregation analysis of *TPP1* gene

Family ID	Primer	Sequence	Temp	Product Length
G-H	Forward primer	GGTGGGGTGAGTTGTAAGGT	62.7^{0}C	360bp
G-H	Reverse primer	TGAGCCAGGTCTGAGTCATG	64.3 ^{0}C	

Table 3.4 Primer sequences used for Co-segregation analysis of *CLN6* gene

Family ID	Primer	Sequence	Temp	Product Length
I	Forward primer	TGTGACCAGAGAAGCCAGAA	63.2 ^{0}C	661bp
I	Reverse primer	CACACACACACACACACGAA	63.2 ^{0}C	
J	Forward primer	ACACAGGAAGGGATGCCAG	65.2 ^{0}C	
J	Reverse primer	CAGAGACCGAGAGCATGAGT	63.2 ^{0}C	416bp

Table 3.5 Primer sequences used for Co-segregation analysis of *MFSD8* gene

Family ID	Primer	Sequence	Temp	Product Length
K	Forward primer	TGGTGTGGCTTGTTTCCTTT	65.2 ^{0}C	416bp
K	Reverse primer	TGTGTGGTCTGGGAAATGAC	62.3 ^{0}C	

3.10 Sanger Sequencing

Amplification reactions were made in a total volume of 25µl, by using 50 ng of DNA, with standard FastStart PCR reagents (Roche), on an ABI Veriti Thermal Cycler (Applied Biosystems).PCR products were purified using Exo-SAP (Exonuclease I and Shrimp Alkaline Phosphatase; incubated at 37°C for 15 min followed by inactivation by heating to 80 °C for 15 min) and sequencing PCR was performed bi- directionally using Big Dye Terminator Ready Reaction Mix kit version 3.1 (Applied Biosystems) and analysed on an ABI 3730xl capillary sequencer. Electropherograms were made on the Sequencer soft- ware to compare sequences of probands versus parents or healthy controls.

3.11 Enzymatic PCR Clean up

Exo-Sap was mixture of Fast-AP, an Alkaline phosphatase which eliminates unused dNTPs, besides Exonuclease I which confiscates ssDNA from PCR products (Primer dimers was not detached.) Enzymes were stored at -20C.The enzyme mix "Exo-fast" was prepared in an Eppendorf, 1ml at a time to minimize freeze thaw with constituents: fifty Exo I (neat), 200ul Fast-AP (neat),750ul water, respectively. It was gently mixed and 7ul of polymerase chain reaction product was transferred to hygienic plate. 2.8ul Exo-Fast was added into product and pipette-mixed with the help of a pipette. Lid was placed and sealed firmly to be run on thermal cycler 37° for 30min, 80° for 15 minutes.

3.12 Preparing Sequencing Reaction

4.5 ul H_2O, 2 ul sequencing buffer, 1 ul primer (forward or reverse), 0.5 ul big dye, and 2 ul cleaned PCR product were used in the sequencing reaction (added last). This mixture was centrifuged briefly and was then run on a thermal cycler for one minute at 94 °C, followed by 25 cycles of 94 °C for 30 seconds, 50 °C for 15 seconds, and 60 °C to four minutes (Reema>Sao>seq protocol). It was stored at +4 ° in the refrigerator overnight. Sephadex cleaning and analysis was done on the next day at 94 °C for 1 min and 24 cycles of 94 °C -1 min, 50 °C- 15 seconds ,60 °C – 4 mins, 4°C. In Sanger Sequencing Workflow the total 15 ul was taken in each amplification reaction, 7.5ul Fast-start Master Mix 5ul H_2O and 0.75ul forward primer (diluted to 10uM), 0.75ul reverse primer (Diluted to 10uM) 1ul DNA (at least 25-50ng/ul).

Table 3.6 Different temperature and time period for PCR Cycles

65° to 55° as below 94 °C-10mins	
	94 °C-30sec
8 Cycles of:	94°C-30sec
	72 °C -45sec
16 Cycles of:	94 °C-30sec
	65 °C-30sec *add a TD option of -0.7 °C
	72 °C -45sec
16 Cycles of:	94 °C-30sec
	55 °C-30sec
	72 °C -45sec
72 °C 5-10min (final extension) 4 °C –forever	

Master mix was made in an Eppendorf tube and distributed into wells (14ul into each), except DNA (1ul) which is added last and then centrifuged briefly and run-on thermal cycler.

3.13 Sequencing Clean-up with Prepared Sephadex Plate

In order to grade DNA, Sephadex G-50 Bioreagent was used, along with fine Corning® FiltrEXTM 96 fine filter plates, 0.66 mm glass fibre filter, polystyrene, and autoclaved distilled water (instead of MilliQ). Sephadex was prepared for each plate of about 50mL tube containing 40ml distilled water with 2.9grams Sephadex. Sephadex was mixed and allowed to hydrate for at least 30 minutes at room temperature. Purification plate was prepared by adding 350ul of the fully mixed Sephadex to individuals wells of a corning glass plate. The glass plate was placed in an empty collection plate and centrifuged both for 3 minutes at 750xg. New plate was placed on a glass plate and entire volume was pipetted for sequencing reaction (10ul) from Sequencing Plate onto the Sephadex columns. It was centrifuged for 5 minutes at 910xg (final volume after spinning should be ~10ul). After placing the plate into sequencer for overnight, results were analysed the following day.

3.14 Bioinformatic: Data Retrieval and Homology Modelling

Basic modelling methodology was followed to model CLN5 based on a single template (PDB ID: 6R99_A) (Table 5.1). For MSFD8, multiple templates were used given in table 5.1. Template search was performed using PSI-BLAST against protein databank (PDB). After the selection of template structure, the query sequence was aligned with the template structure in Chimera Integrated MODELLER (Pettersen *et al.*, 2004). Default parameters were opted for homology modelling, generating 10 models. Models prepared by UCSF Chimera were then evaluated based on the DOPE and ZDOPE scores (Pieper *et al.*, 2006; Shen & Sali, 2006). Models with lowest DOPE and ZDOPE scores were subjected to energy optimization to relieve the structures from clashes, overlaps, and faulty interactions with atoms among residues. For energy optimization of models, steepest gradient method was used in Chimera and structure quality assessment analysis were done in MolProbity.

4. RESULTS

4.1 Initial clinical information of all Families

4.2. Family-A

Family A belongs to Khyber Agency, Pakistan and members includes four siblings, two of which were affected from consanguineous parents (Fig.4.1). Patient IV.1 is a female and patient IV.2 is a male with different ages. Mother, father and two sisters are healthy and unaffected. Based on initial investigation, all the affected individuals were showing the symptoms of CLN5 such as memory loss, visual impairment, behavioral abnormalities (wandering, episodes of laughter, agitation) and less to no speech and seizures. All reports including their MRI was collected at the time of initial information. Medical data has been compiled in Table 4.1. In genetic analysis of family- A, whole genome SNP mapping from 2 affected family members was done in UCL, UK. The NGS of a single affected individual in the family identified a candidate with novel homozygous mutation in *CLN5*. The innovative homozygous missense variant *ENST00000377453.8*: c.477T>C, p. Cys159Arg (chr13: 76,996,037) was identified in *CLN5* gene in family A. This mutation occurred inside the most important homozygous block (chr13: 73,075,970-77,070,727) in exon 4 (Fig.4.3). Rest of family members were found to be heterozygous carriers. Electropherograms characterize the locations of mutated carrier and normal sequences (Figure 4.1.1).

To obtain co-segregation results, the primers were designed (tables given in section materials and methods) and study was done for other affected and normal individuals. This proved that all affected individuals are homozygous for transformation c.477T>C, p. Cys159Arg and normal are heterozygous. The clinical examination was properly done in hospital and confirmed by specialist about the status of disease. Homozygosity mapping of family A analysis depicting the homozygous block (in green box) where the reported variants were identified (Fig 4.5). Initial clinical assessment of all families was done by Professor Dr Tipu Sultan (FCPS Pediatric Neurologist Children Hospital, Lahore) and by Professor Dr. Henry Holden University College, London. In this family the affected individual IV.1 is a ten-year-old female child and second is a seven-year-old male. After a standard achievement of formative achievements, both siblings experienced evident psychomotor relapse. She additionally developed visual weakness and ophthalmologic

evaluation uncovered respective optic circle paleness. In IV.1 patient, critical cognitive decline and status of speech was seriously impaired, mostly based on incoherent words. Actual assessment at ten years more uncovered cerebellar signs (ataxia dysmetria). Similarly, the other patient IV.2 showed irregularities, cognitive decline, and hindered night vision at six years old (Table 4.1.1). The proband visual impairment progressed to blindness and funduscopic investigation discovered bilateral optic disc pallor. In the next months, patient-1 experienced frequent falls and was unable to walk, regardless of being supported or not. At seven years, patient IV.1 was simply ready to creep and sit unsupported. His speech was restricted to babbling and neurological inspection pointed towards showed to ataxia and tremors.

The two kin experienced typical and severe myoclonic seizures, beginning at the period of 7.5 years and 6 years for both IV.1 and IV.2. EEG showed multifocal epileptic form releases. In patient IV.1, brain MRI showed (Figure 4.4 A) diffused cerebellar and cerebral atrophy. MRI found abnormalities in patient 2 containing enlarged ventricles and subarachnoid spaces, cerebellar atrophy with prominent folia, and white matter hyper intensities with predominant involvement of the posterior limb of the inner capsule. Patient IV.1 expired at eleven years and patient IV.2 is alive while, September 2022 and presents refractory epilepsy, severe cognitive decline, and ataxia.

4.3 Family-B

Family B belongs to Gujrat, Punjab Pakistan. This family consist of 5 members which revealed an affected male, born by consanguineous parents with both normal parents and siblings (Figure 4.2). The initial symptoms of NCL were clearly diagnosed after examination. The affected boy IV.4 revealed by decline of earlier attained skills; subsequent initial seizure at the age of sixyears. Before the occurrence of these defects, being development was normal and milestones had been reached age-appropriately. All medical reports and other clinical details with the MRI were collected at the time of collection of initial information. Initial information was collected and summarized (Table 4.2). Co-segregation results, and primers were designed. Homozygosity mapping of family B analysis also shown in (Figure 4.3) where the reported variants were identified.

The novel CLN5 frame-shift variation ENST00000377453.8: c.925 926del, p. Leu309AlafsTer4 was found in this family (chr13: 77,000,816). Exon 4's deletion of 2

nucleotides at position c.925 926 is predicted to result in a frame-shift. All affected individuals are homozygous for the mutation c.925 926del, p.Leu309AlafsTer4, according to a co-segregation analysis, while normal individuals are heterozygous. The significant impact of these CLN5 mutations was established using in-silico analyses (including SIFT and Polyphen). Family B includes a ten-year-old boy who was born of consanguineous parents and had a decline in previously acquired skills after experiencing his first seizure at the age of six. Before appearance of first symptom, development was normal, and achievements had been reached at an appropriate age. Myoclonic jerks were the first symptom that was noticed, and they increased in frequency throughout the first year. He also developed focal impaired awareness motor seizures at the age of seven, which developed into bilateral to nucleonic seizures by the time he was nine years old Seizures are resistant to drug treatment. In parallel with seizure progression, a deterioration of motor, verbal, and memory functions were reported. Motor deterioration manifested as an uneven gait and progress into an absolute inability to work properly since last 2 years. Although the patient was still able to stand and sit without support, her vocal abilities were completely paralyzed (her language was limited to one or two words), exactly like her memories. The family also noted a vision impairment throughout the most recent year (2019), with the inadequacy to fix objects or faces. Frothing at the mouth excessively, axial ataxia with positive cerebellar symptoms (dysmetria, incoordination, no nystagmus), decreased muscular tone, typical reflexes, and an upbeat grower were all noted in a young child's neurological examination. Cerebellar atrophy was observed by brain MRI, and the EEG showed multifocal epileptiform discharges. (Figure 4.4 B).

4.4 Family-C

Family C belongs to Haripur and consists of 4 siblings, 3 of them is affected. Parents and one brother are healthy and unaffected (Figure 4.6). Afterward a normal attainment of developmental milestones, practiced obvious psychomotor regression. With behavioral fluctuations and memory loss, tracked by regular falls. Patient also developed visual impairment, behavioral abnormalities memory loss. All reports and other details with the MRI were collected at the time of collectionof initial information. Initial medical information was together and summarized in (Table 4.3). NGS sequencing of a single affected individual in the family identified a candidate homozygous mutation with a stop gained homozygous variation in *CLN5*.We found the reported *CLN5* stop gained variant *ENST00000650992.1*:

c.524T>A, p. Leu175Ter (chr13: 77,570,074). Both parents were found to be carriers. Electropherograms characterize the sites of mutated, carrier and normal sequences (Figure 4.6.1). For co-segregation study major primers were considered and examination was done for other affected and normal persons.

Detail clinical evaluation was summarized in (Table 4.3.1).In patient, regression in progress at the age of 6 years with behavioral variations and memory loss,surveyed by normal falls. She also established visual impairment and ophthalmologic assessmentrevealed bilateral optic disc pallor. The girl presented significant cognitive decline and her speech was severely impaired. Patient started to show behavioral abnormalities memory loss. EEG showed multifocal epileptiform discharges.

4.5 Family -D

Family D belongs to Karachi, Pakistan, and consist of 3 family members. Both parents are healthy and normal, but the daughter IV.1 is affected (Fig. 4.7). In 2015 this case was reported in Agha khan hospital with complaints of regression of milestones 3 years of age and seizures. Proband was the only child congenital to consanguineous parents who were first cousins. The patient also had issues of not being able to sit in one place and aggressive behavior (Table 4.4)Patient was born term appropriate for gestational age mother had one ectopic pregnancy prior to her birth. Initial medical information was summarized in (Table 4.4.1). For co-segregation analysis primer was designed and analysis was done for other affected and normal persons.

Co-Segregation analysis proved that all affected individuals are homozygous mutant for mutation c.616C>T, p. Arg206Cys normal is heterozygous carrier (Figure 4.7.1). The clinical examination was properly done in hospital and conformed by specialist about the status of disease. Initial clinical examination was done by Professor Dr. Tipu Sultan (FCPS Pediatric Neurologist Children Hospital Lahore) and by Professor Dr Henry Houlden university College London. Family D consist of 3 members. This is an eight years old girl who first visited Aga Khan Hospital at the age of 4 years, with the complaints of regression in achieving milestone after 3 years of age and seizures for the last 7 months. At the age of 3, patient started regression in her milestones and her conditioned progressively advanced with complains of inability to stand or walk (September 2014) seizures episodes lasted for about one minute characterized with lip twitching and up-rolling of eye. Sodium valproate was

prescribed at this time. Proband developmental milestones showed that her ability to support her neck was stable at 3 months of age she started sitting at 6 months, speech was delayed and started saying single words. At the age of 3 patient was reported to be unable to sit in one place and showed patterns of aggressive behavior. There was cranial nerve involvement and there was one hypo pigmented spot on the abdomen.

An EEG was already done which was suggestive of non-convulsive status epilepticus Initial impression was a neurodegenerative disorder Like Neuronal ceroid lipofuscinosis like epileptic encephalopathy. A repeat EEG showed more focal epilepsy with bilateral temporal-occipital discharges which were very frequent. A repeat MRI showed diffuse volume loss. Over the next one-year multiple seizures were noted with very poor control on multiple drugs. Patient was diagnosed with as intractable epilepsy and folic acid, B6 was added in her medication. Her health further regressed and she was bed bound with additional behavioral changes such as no eye contact, no speech, drooling and later some tremor along with dystonia was seen in her upper limbs at movement. Feeding became difficult and a nasogastric tube was required for feeding. Patient later underwent a gastrostomy and fundoplication was also done as there was reflux consequently aspirations. Unfortunately, patient expired in January 2019 due to sepsis, and acute gastroenteritis with severe dehydration.

4.6 Family-E

Family E is from Sialkot, Pakistan and this family consists of two siblings, both of them are affected. They are born to consanguineous parents. The patient IV.1 and IV.2 both are females. The affected individual is from IV generation (Table 4.5) and born to consanguineous parents with an autosomal recessive pattern of inheritance (Figure 4.8). Both, parents and one brother, are healthy and unaffected. Homozygous mutations in *TPP1* were identified in the Family E. In this family, the homozygous missense variant ENST00000299427. 12: c.616C>T, p. Arg206Cys (chr 1166,38,277) was identified in *TPP1*. Parents and siblings were heterozygous carriers. Electropherograms signify the positions of mutated, carrier and normal sequences (Figure 4.8.1).

For co-segregation study, a basic primer was designed and examination was done for other affected and normal individuals. Co-segregation investigation proved that all affected entities are homozygous mutant for mutation c.616C>T, p. Arg206Cys, unaffected members

are heterozygous carrier. Patient is a five years old female, who at 3 years of age showed symptoms and was later diagnosed with epilepsy, Motor, regression, Global regression, decrease cognition apparent psychomotor regression. It was later followed with behavioral changes, such as hearing loss, affected speech (Table 4.5.1) and eyes when awake were held and semi co-operative.

Neurological assessment such as; increased tone, power include 4-5/5, increased reflexes. The results of MRI showed cerebral atrophy with foci of demyelination in periventricular white matter and EEG was multifocal epileptiform activity and slow waves in a generalized. Ophthalmologist suggested that fix and follow light and DBD with epilepsy. Ventricular and extra ventricular CFS space are dilated.

4.7 Family-F

Family F belongs to Riwand, Pakistan and consists of five family members, two of whom are affected. Patient IV.4 and IV.5 both are females. The affected individual is from 4th generation and born to consanguineous parents with an autosomal recessive pattern of inheritance (Figure 4.9) after a standard progress in learning basic behavioral responses to external stimuli, both siblings experienced apparent psychomotor regression, behavioral abnormalities and additional symptoms commonly observed in CLN2 disease (Table 4.6). Homozygous mutations in *TPP1* were identified in the family F. In family F, the homozygous missense variant *ENST00000299427.11*: c.622C>T, p. Arg208Ter (chr1166,38,271) was identified in *TPP1*. Parents were found to be heterozygous carriers. Electropherograms signify the sites of mutated, carrier and normal sequences (Figure 4.9.1).

The primers were designed for co-segregation analysis, and the final study was conducted on both affected and unaffected individuals. All affected individuals are homozygous mutant for the mutation c.622C>T, p.Arg208, according to co-segregation data. Heterozygous carriers are the members who are unaffected. Proband 1 is a female who is seven years old, whereas Proband 2 is a female who is three years old. Regression started in patient 1 at the age of 4 and was characterized by behavioural abnormalities, no speech, no vision, and normal hearing. EEG showed the background disturbance and with multifocal generalized Epileptiform discharges. Neurological Assessment revealed OFC: 47.5cm, GCS: 7/15, Hyperreflexia, Hypertonia, Planters, up going. Similarly, patient 2, a 3-year-old girl started to show behavioral abnormalities, memory loss, with visual weakening progressing to

blindness and funduscopic examination revealed bilateral optic disc pallor. Clinical data shows no speech and low vision. At the ages of five and six for patient 1 and two years for patient 2, respectively, both siblings experienced frequent and severe myoclonic seizures. EEG shown multifocal epileptiform discharges (Table 4.6.1). In patient 1, brain MRI shown diffuse cerebellar and cerebral atrophy. Patient 1 deceased at the age of 10 years. Her sister is presently alive.

4.8 Family-G

Family G belongs to Multan and consists of two siblings born to consanguineous parents, one of whom is affected IV.2. The parents of proband are healthy and unaffected. Family was traced back to generations and shows an autosomal recessive pattern of inheritance (Figure 4.10). The affected male member from fourth generation was (IV.2) on examination the patient was showing different sign and symptoms of CLN2 disease. On examination the history of the patient indicated in proper developmental regression (Table 4.7). Homozygous mutations in *TPP1* were identified in the Family G. In family G the homozygous missense variant *ENSP00000299427.6 NM_000391.3p.*Glu402Gly c.1205A>G (chr 11-66,36,734) was identified in TPP1. Parents and family were found to be heterozygous carriers. Electropherograms characterize the locations of mutated, carrier and normal sequences (Figure 4.10.1).

For co-segregation analysis the primers were made and analysis was achieved for other affected and normal persons. Co-segregation results showed that all affected individuals are homozygous mutant for mutation Glu402Gly c.1205A>G unaffected is heterozygous carrier. The clinical examination was properly done in hospital and confirmed by specialist about the status of Disease (Table 4.7.1). Initial clinical assessment was done by Professor Dr. Tipu Sultan (FCPS Pediatric) Neurologist Children Hospital Lahore) and by Professor Dr. Henry Houlden University College London. Patient is a seven years old male. Patient history shows developmental regression which started at the age of 4 years with behavioral changes and memory loss, surveyed by regular falls. He also established seizures at 3 years of age with a maximum frequency of 6-7 episodes and CNS with encephalopathy. His speech was severely impaired and he started to show behavioral abnormalities, memory loss. EEG showed focal epileptiform activity. By investigation the MRI of the brain showed deep white matter and perpendicular hypertensive on T2W.

4.9 Family -H

Family H belongs to Jhelum, Pakistan and consists of 5 family members, one of whom is affected. The affected individual is female IV.3 from fourth generation (IV) and born to consanguineous parents with an autosomal recessive pattern of inheritance (Figure 4.11). Both parents are healthy and unaffected. The height of patient is 98 cm and weight are 12.5kg, head size is normal and blood pressure is hypertension. All medical examination has been done by medical experts (Table 4.8). Homozygous mutations in TPP1 were identified in the Family H. In H family, the homozygous missense variant *ENSP00000494324. 1: ENST00000643342.1 p*:Ter93TrpextTer3: c.278A>G was identified in TPP1. Both, parents and siblings were heterozygous carriers. Electropherograms located the mutated, carrier and normal sequences (Figure 4.11.1). The primers were designed and study was done for other affected and normal persons. Co-segregation analysis proved that all affected individuals are homozygous mutant for variation Ter93TrpextTer3: c.278A>G unaffected members are heterozygous carrier. The clinical examination was properly done in hospital and conformed by specialist about the status of Disease. Initial Clinical assessment was done by Dr. Faisal Zafar (MD, FCPS Children Hospital Multan) and by Professor Dr. Henry Houlden University College London. Family H having a 4 years old affected female. On proper clinical examination found CNS involves encephalopathy and severe speech problem. In proband, regression was progressive around the age of 3 years with behavioral fluctuations and seizures (Table 4.8.1). Seizures started at the age of 2, lasting for about 2 minutes with the frequency of 2-3 episodes. MRI showed mild cerebella follicle prominence.

4.10 Family-I

Family-I resides in the province Punjab, Pakistan. As presented in pedigree, family comprises of over four generations with 14 members, 6 deceased and 8 living. It shows an autosomal recessive pattern of inheritance (Figure 4.12).The affected male member from fourth generation was (IV.5) conceived from a consanguineous marriage and a male sibling (IV.2) was reported to have expired after three days of birth. In diseased family member IV.2, the suspected cause of death was the presence of fluid in brain (Table 4.9).

However, medical reports were not available for family members IV.1 and IV.5. The female sibling (IV.5) had complaints of leg bending (not always but sometimes). Whole genome

SNP mapping using DNA from 1 affected family members identified a region of homozygosity. Homozygous mutations in *CLN6* were identified in the family-I. In family I, the homozygous missense variant *ENST00000249806. 11*: c.768C>G, (Chr 15:68,20,83,08) was identified in *CLN6*. Both parents are heterozygous carriers. Electropherograms signify the locations of mutated, carrier and normal sequences (Figure 4.12.1). The primers were designed for co-segregation and study was done for other affected and normal individua. Co-segregation study proved that all affected individuals are homozygous mutant for alteration c.768C>G and unaffected individual are heterozygous carriers. The patient, a 24-year-old male, first showed symptoms at the age of 12. He was able to walk and sit independently, normally vision and hearing and no behavioral issues, but had ataxia gait, dysarthria, mild intellectual disability and dysphagia (Table 4.9.1). Focal fatty deposit was seen in the vertebral body of DV8.The MRI reports were normal.

4.11 Family-J

Family J belongs to Sahiwal, Pakistan and consists of two siblings, one of them is affected IV.2. They were the primary and second-born to consanguineous parents (Figure.4.13). Patient is a male. Aftera normal examination experienced evident regression of milestones. Both parents were healthy. At the time of initial examination, the affected individual was revealing the visible signs of CLN6, no/less speech and seizures. All reports and other details with the MRI were collected at the time of collection of initial information. Initial medical information was summarized (Table 4.10). Homozygous mutations in *CLN6* were identified in the Family J. In family J, we found the in deletion *ENST00000249806.11: p.* Ser265del, c.794_796delCCT (chr1568,500,617) in *CLN6*. Both parents were found to be heterozygous carriers. Electropherograms characterize the positions of mutated, carrier and normal sequences (Figure 4.13.1). Detail Clinical evaluation was summarized in (Table 4.10.1). For co-segregation investigation specific primers were made and studywas done for other affected and normal persons. Co-segregation analysis proved that an affected individual is homozygous mutant for transformation p. Ser265del, c.794_796delCCT unaffected is heterozygous carrier. The clinical examination was properly done in hospital and confirmed by a specialist about the status of Disease. Neuroimaging abnormalities in patient involved as a substitute of enlarged ventricles and subarachnoid spaces, cerebellar atrophy with projecting folia, and white matter hyper intensities with chief contribution of the subsequent limb of the core capsules.

4.12 Family-K

Family K belongs to Faisalabad, Pakistan and involves three siblings with two affected. They are the from consanguineous parents (Figure. 4.14). Patient IV.2 and IV.3 both are boys. Mother, father and one sister are healthy and unaffected. During the initial investigation, individual was showing the signs of *MFSD8* including delay in development, delay regression visual impairment, seizures. All reports and other details with the MRI were collected at the time of collection of initial information. Initial medical information was together and summarized in (Table 4.11). Clinical details were shortened in (Table 4.11.1). Whole genome SNP mapping using DNA from 2 affected family members was done in UCL, UK. This identified a region of homozygosity NGS of a single affected individual in the family identified a candidate homozygousmutation with a novel homozygous alteration in *MFSD8* gene. In this family, the novel homozygous missense variant *NM_152778.2 c.938T>G* p.Leu313Arg (chr4:12,88,51,898)was identified in *MFSD8* gene.

Electropherograms characterize the positions of mutated, carrier and regular sequences (Figure 4.14.1). For co-segregation study a set of primers was made and examination wasdone for other affected and normal persons. In family (K) the patient IV.1 is an 8-year-old male and patient 2 is a 6-year-old male. Afterward a normal achievement of developmental deterioration at the age of 3.5 years and initial association was with white matter. In patient IV.2, behavioral changes, memory loss, dementia, also tracked by frequent falls with encephalopathy. In IV.2 patient facial dysmorphism include the height of 101cm, weight 13kg, no head control and MRI reports contain extravehicular space, cerebral and cerebellar atrophic changes. Similarly, patient IV.3 started to show developmental regression, seizures with the maximum frequency 5, and duration about 25 min. facial dysmorphism include the height of IV.3 patient 112cm, weight 12kg, severe speech problem, EEG with diffuse encephalopathy. The Brain MRI contain prominent cerebellar folia and extra ventricular space most likely due to neuronal degeneration. Both patients were also followed by dementia.

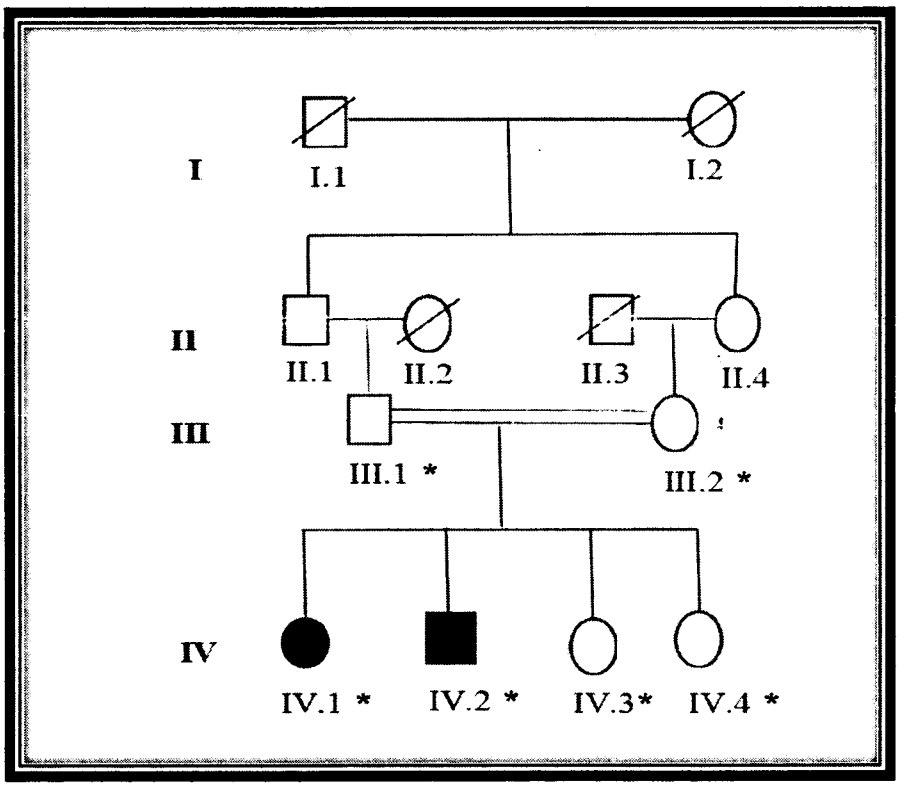


Figure 4.1 Detailed family pedigree of Family **(A)** which is clearly showing the autosomal recessive mode of inheritance. The affected individuals are IV.1. and IV.2. The healthy family members are III.1(father) III.2 (mother), IV.3(sister). IV.4(sister). (*) means that the blood of these members was available at the time of collection

Table 4.1 Initial Clinical Symptoms of Members of Family **(A)**

ID	Gender	Status	Age	Initial Clinical Findings			
				Speech	**Memory loss**	**Seizures**	**Behavioural Abnormalities**
III.1	Male	Normal	29years	Normal	No	No	No
III.2	Female	Normal	24years	Normal	No	No	No
IV.1	Female	Affected	10years	Normal	Yes	Yes	Severe
IV.2	Male	Affected	7years	Normal	Yes	Yes	Severe
IV.3	Female	Normal	5years	Normal	No	No	No
IV.4	Female	Normal	2.6years	Very Limited	Yes	Yes	No

Table 4.1.1 Detail Clinical Evaluation of Family **(A)**

Family A	Patient ID IV.1	Patient ID IV.2
Gender	Female	Male
Age years	10 years	7 years
Status	Affected	Affected
Visual impairment	Yes	Yes
Seizures	Yes	Yes
Psychomotor regression	Yes	Yes
Memory loss	Yes	Yes
Speech	Limited	Severely affected
Ophthalmologic	Bilateral optic discpallor	Bilateral optic disc pallor
Behavioral Abnormalities	Sever	Sever
EEG	Multifocal epileptiform discharges	Multifocal epileptiform discharges
MRI	Diffuse cerebellar and cerebral atrophy	Cerebellar atrophy with prominent folia, and white matter

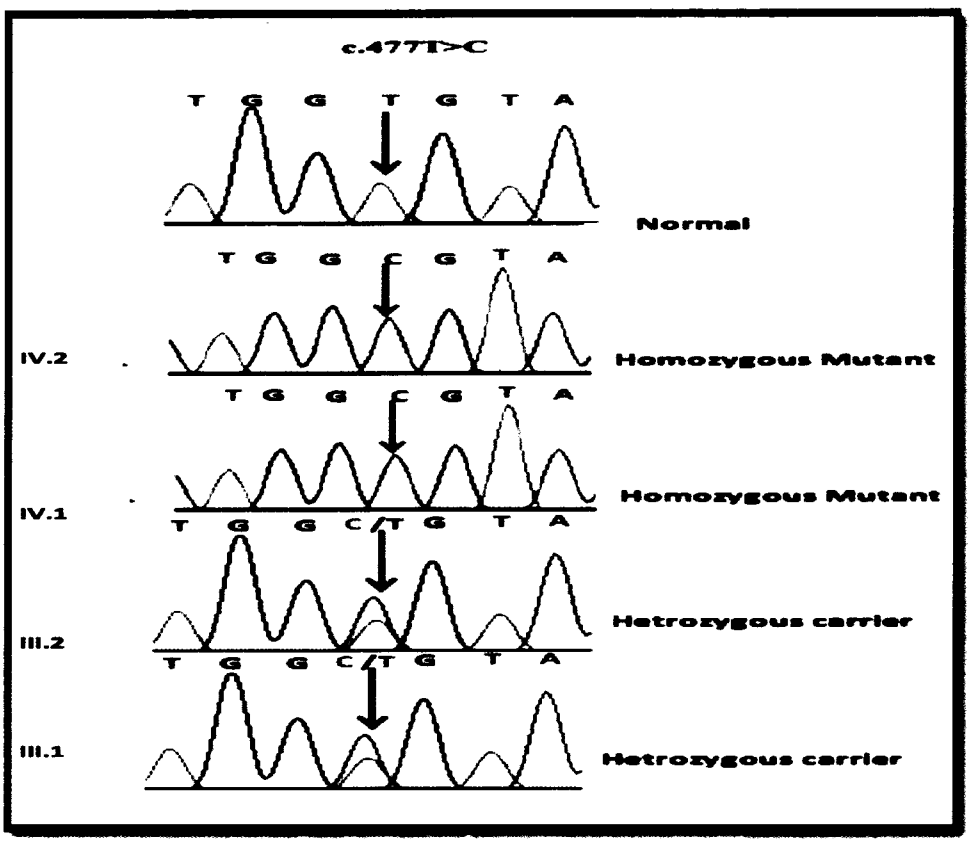


Figure 4.1.1 Variants and Sanger sequencing electropherogram confirming the variants in the families **(A).** IV.1 and IV.2 both are probands which are homozygous Mutant. Both parents are heterozygous carrier with consanguineous marriage. The peaks in electropherograms are clearly showing the mutation at the position c. 477T>C.

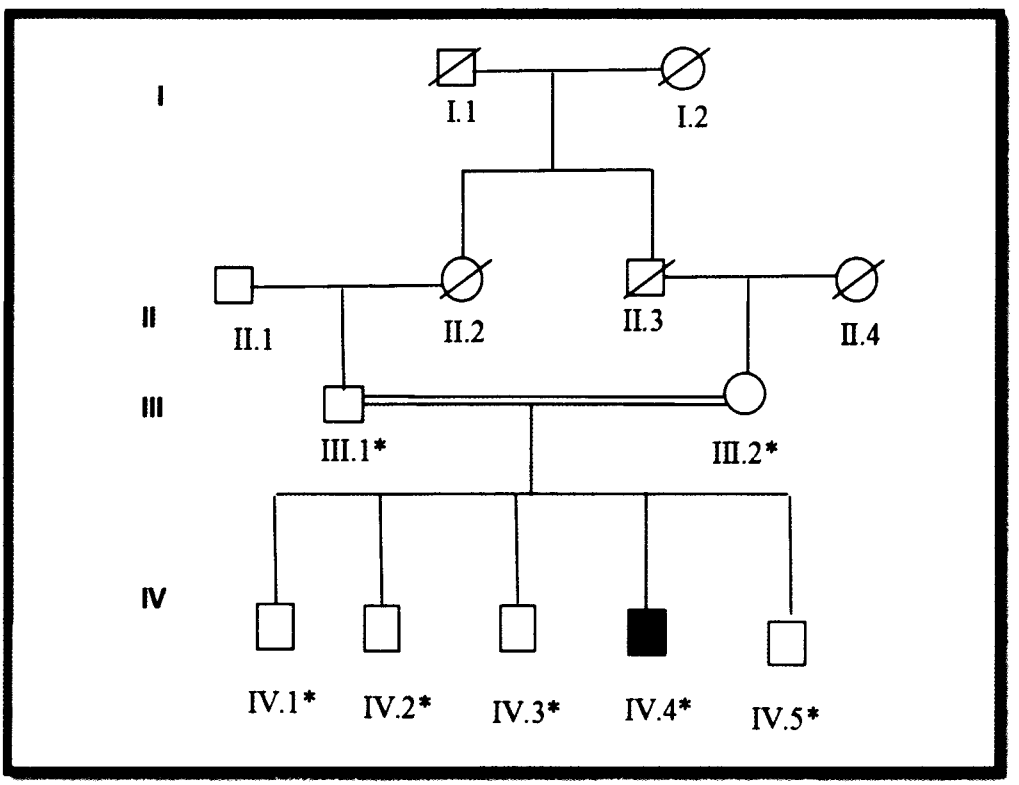


Figure 4.2 Detailed family pedigree of Family **(B)** which is clearly revealing the IV.4.the autosomal recessive mode of inheritance.III.1 (father), III.2(mother), IV.1(brother),IV.2 (brother), IV.3(brother).Autosomal Recessive mode of inheritance are found in IV generation.

Table 4.2 Detailed Initial Clinical Symptoms of Members of Family **(B)**

ID	Gender	Status	Age	Initial Clinical Findings				Location
				Speech	**Memory loss**	**Seizures**	**Behavioural Abnormalities**	
III.1	Male	Normal	49years	Normal	No	No	No	Gujrat
III.2	Female	Normal	36years	Normal	No	No	No	Gujrat
IV.1	Male	Normal	14years	Normal	No	No	No	Gujrat
IV.2	Male	Normal	12years	Normal	No	No	No	Gujrat
IV.3	Male	Normal	10years	Normal	No	No	No	Gujrat
IV.4	Male	Affected	8years	Very Limited	Yes	Yes	Severe	Gujrat
III.5	Male	Normal	5months	Normal	No	No	No	Gujrat

Table 4.2.1 Detail Clinical Evaluation of IV.4 Affected Member of Family **(B)**

Family B	Patient ID IV.4
Gender	Male
Age years	8 years
Status	Affected
Visual impairment	Yes
Seizures	Yes
Psychomotor regression	Yes
Memory loss	Yes
Speech	Very limited 1-2 words
Ophthalmologic	Bilateral optic disc pallor
Behavioural Abnormalities	Yes
EEG	Multifocal epileptiform discharge
MRI	Diffuse cerebellar and cerebral atrophy

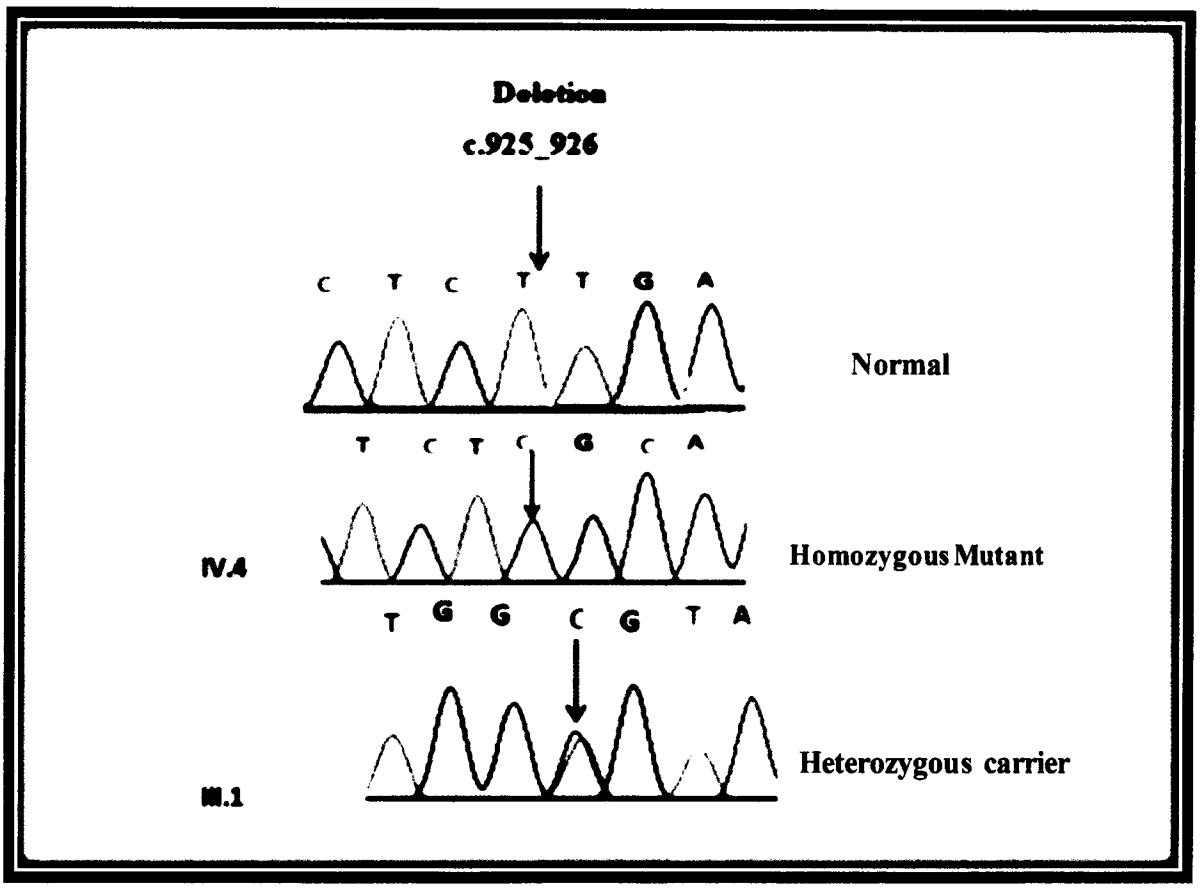


Figure 4.2.1 Sanger sequencing electropherograms validating the variants in the families (B). IV.4 is proband which is homozygous mutant, III.1 is a healthy individual which is heterozygous carrier. Electropherograms Clearly showing the position of deletion at c.925_926.

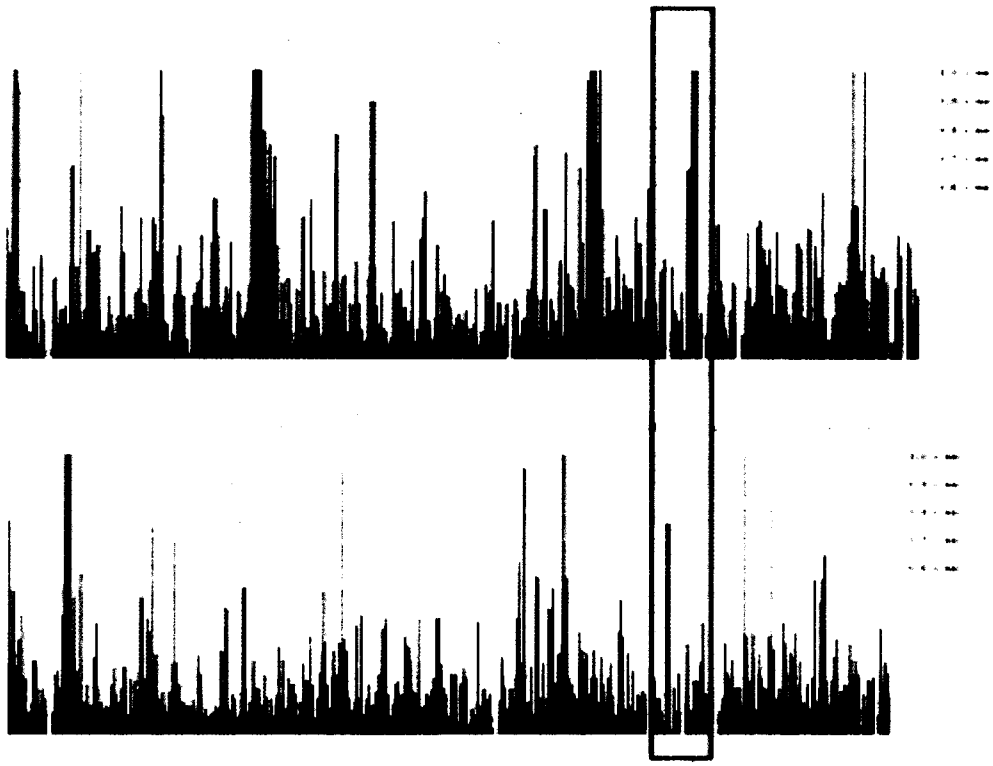


Figure 4.3 Homozygosity mapping of family **A** and **B** analysis representing the homozygous block (in green box on chromosomes 12) where the reported variants were identified.

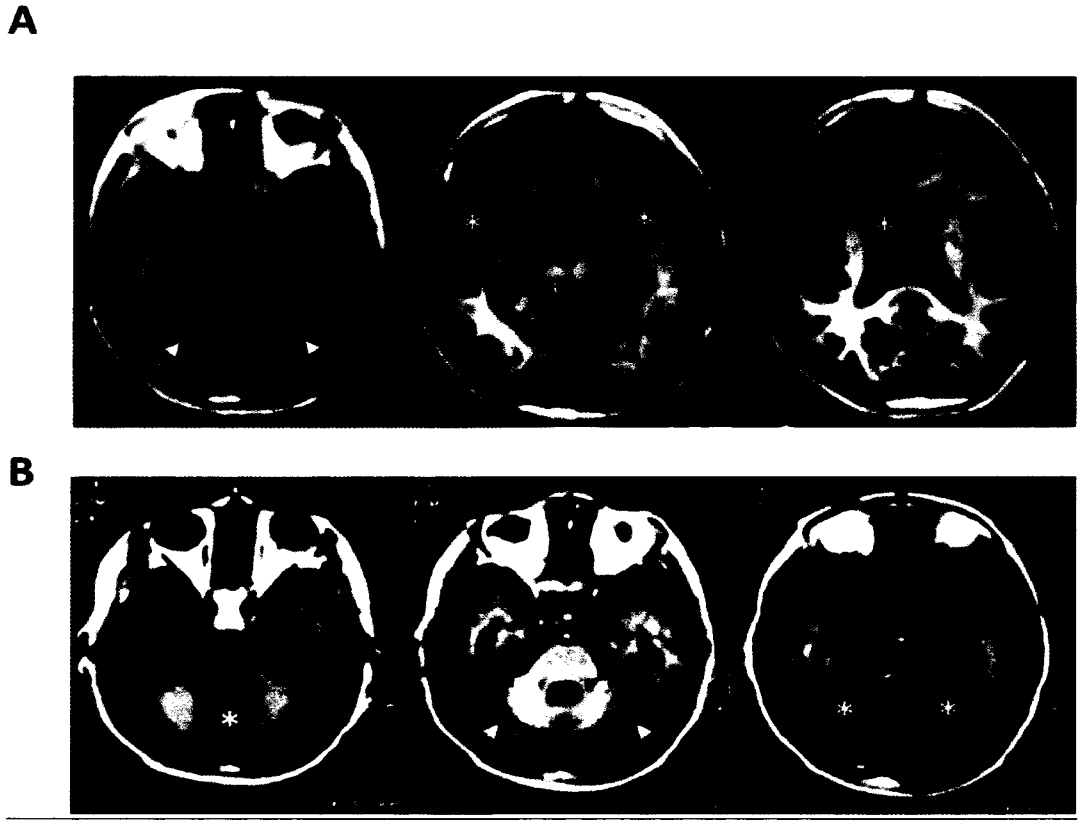


Figure 4.4 Neuroimaging findings. **(A)** Family A (patient 2), axial T1- weighted MRI section show diffuse cerebral and cerebellar atrophy (white arrows), with secondary enlargement of the lateral ventricles, sylvian scissor, and subarachnoid spaces (asterisks). **(B)** Family B, axial T1- weighted scans showing cerebellar atrophy (White arrows) with cortical thinning and fissure enlargement of the vermis and hemispheres(asterisks).

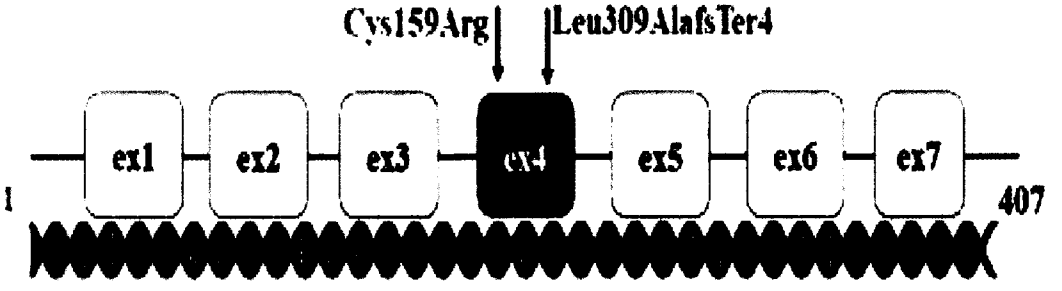


Figure 4.5 The exonic organization of the *CLN5* gene depicting the location (exon 4)of the reported novel variants in family **A** and **B.**

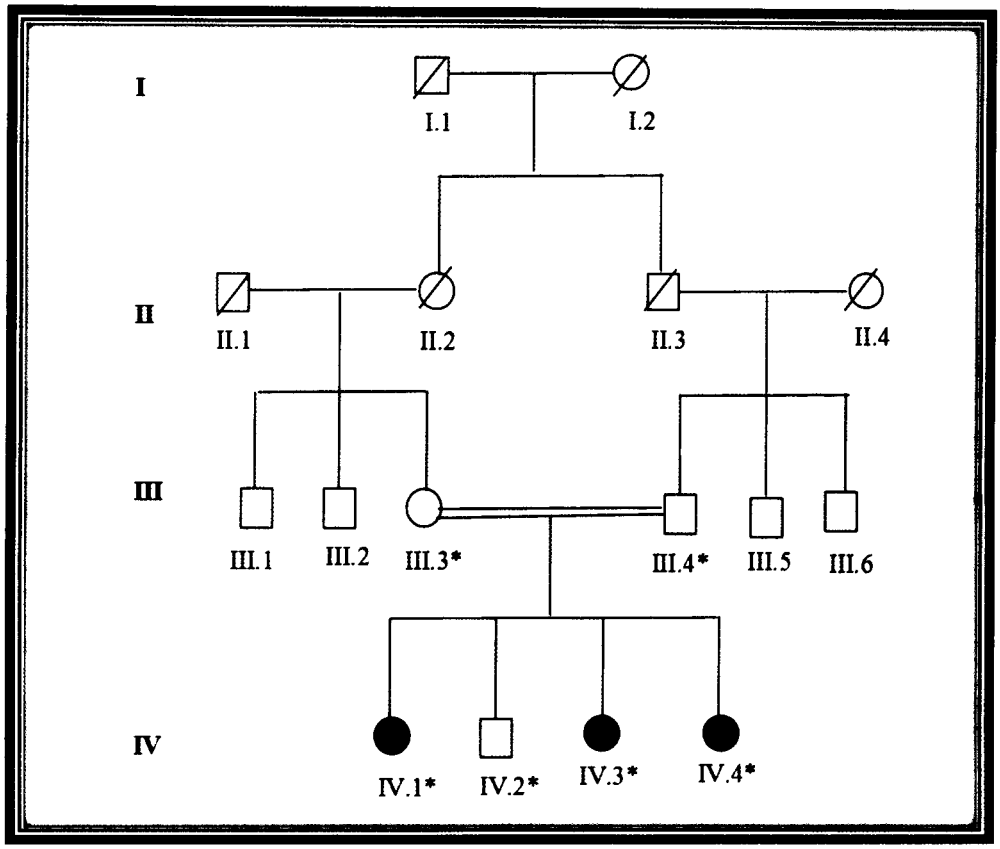


Figure 4.6 Detailed family pedigree of Family **(C)** which is clearly revealing autosomal recessive mode of inheritance. The affected members are IV.1,1V.3,IV.4. The healthy members are III.4(father), III.3(mother), IV.2(brother). (*) means that the blood of these members was available at the time of collection.

Table 4.3 Initial Clinical Symptoms of Members of Family **(C)**

ID	Gender	Status	Age	Initial Clinical Findings				Location
				Speech	**Memory loss**	**Seizures**	**Behavioural Changes**	
III.3	Female	Normal	36years	Normal	No	No	No	*Abt.bd
III.4	Male	Normal	40years	Normal	No	No	No	*Abt.bd
IV.1	Female	Affected	10years	Severely Affected	Yes	Yes	Yes	*Abt.bd
IV.2	Male	Normal	8years	Normal	No	No	No	*Abt.bd

* Abbottabad

Table 4.3.1. Detail clinical evaluation of Family **(C)**

Family C	Patient ID (IV.1)
Gender	Female
Ethnicity	Awan
Age years	10 years
Status	Affected
Visual impairment	Yes
Seizures	Yes
Psychomotor regression	Yes
Memory loss	Yes
Speech	Very limited (1 word)
Ophthalmologic	Bilateral optic disc pallor
Behavioural Abnormalities	Yes
EEG	Multifocal epileptiform discharges
MRI	Diffuse cerebellar and cerebral atrophy

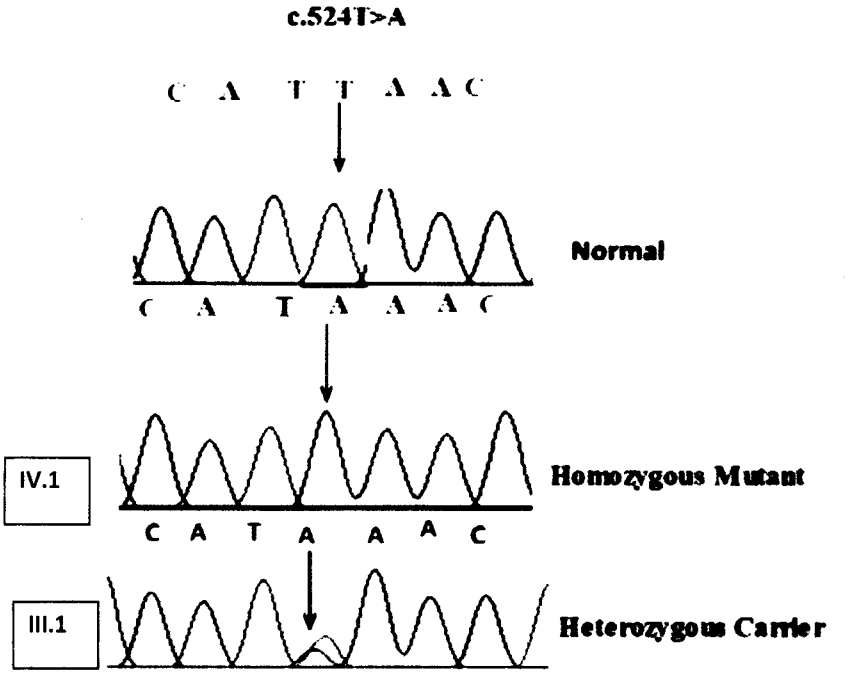


Figure 4.6.1 In Sanger sequencing, electropherograms confirming the mutation in the family **(C)** which is p. Leu175*c.524T>A homozygous stop gained variants.IV.1 is proband which is homozygous mutant, III.3 is a healthy member which is heterozygous carrier.

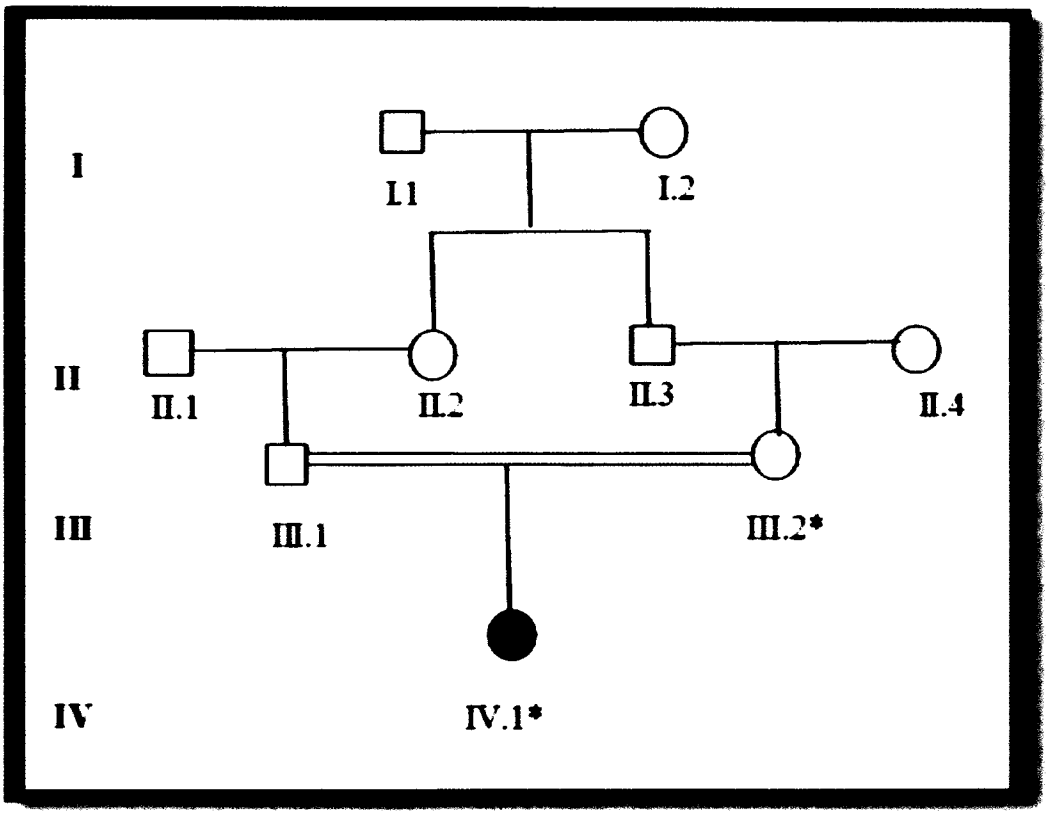


Figure 4.7 Family **(D)** in which IV.1 is a proband, III.1(father), III.2(mother) are healthy family members and clearly showing AutosomalRecessive pattern of inheritance. (*) which means to have blood samples of these family members.

Table 4.4 Initial Clinical Symptoms of Members of Family **(D)**

Family ID	III.1	III.2	IV.1
Gender	Male	Female	Female
Location/Provence	Karachi/Sindh	Karachi/Sindh	Karachi/Sindh
Age	39 years	36years	8years
Status	Normal	Normal	Affected
Consanguineous Parents	Yes	Yes	Yes
Behavioural Abnormalities	Normal	Normal	Aggressive

Table 4.4.1. Detail Clinical Evaluation of Family **(D)**

Family D	Patient ID (IV.1)
Gender	Female
Age years	8 years
Status	Affected
Visual impairment	Yes
Seizures	Yes
Psychomotor regression	Yes
Memory loss	Yes
Speech	Delay
Epilepsy	Yes
Ophthalmologic	Bilateral optic disc pallor
Behavioural Abnormalities	Sever
EEG	Bilateral temporal occipital discharges
MRI	Diffuse cerebellar and cerebral atrophy

ID	Gender	Status	Age	Initial Clinical Findings		
				Consanguineous Parents	**Age onset**	**Location/Province**
III.1	Male	Normal	29years	Yes	N/A	Sialkot /Punjab
III.2	Female	Normal	28years	Yes	N/A	Sialkot /Punjab
IV.1	Female	Affected	5years	Yes	By birth	Sialkot /Punjab
IV.2	Female	Affected	3years	Yes	N/A	Sialkot /Punjab

Table 4.5.1 Clinical Evaluation of Family **(E)**

Family E	Patient ID (IV.1)
Gender	Female
Age years	5 years
Status	Affected
Visual impairment	Yes
Seizures	Yes
Psychomotor regression	Yes
Memory loss	Yes
Speech	Delay
Epilepsy	Yes
Ophthalmologic	Bilateral optic disc pallor
Behavioural Abnormalities	Sever
EEG	Bilateral temporal occipital discharges
MRI	Diffuse cerebellar and cerebral atrophy

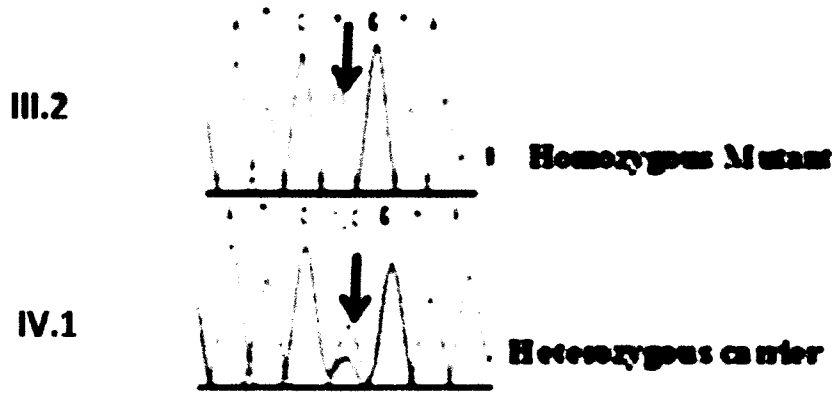


Figure 4.8.1 Variants and Sanger sequencing electropherograms endorsing the variants in the family **(E)**. The homozygous missense variant c.616C>T, p. Arg206Cys is identified with *TPP1* gene.

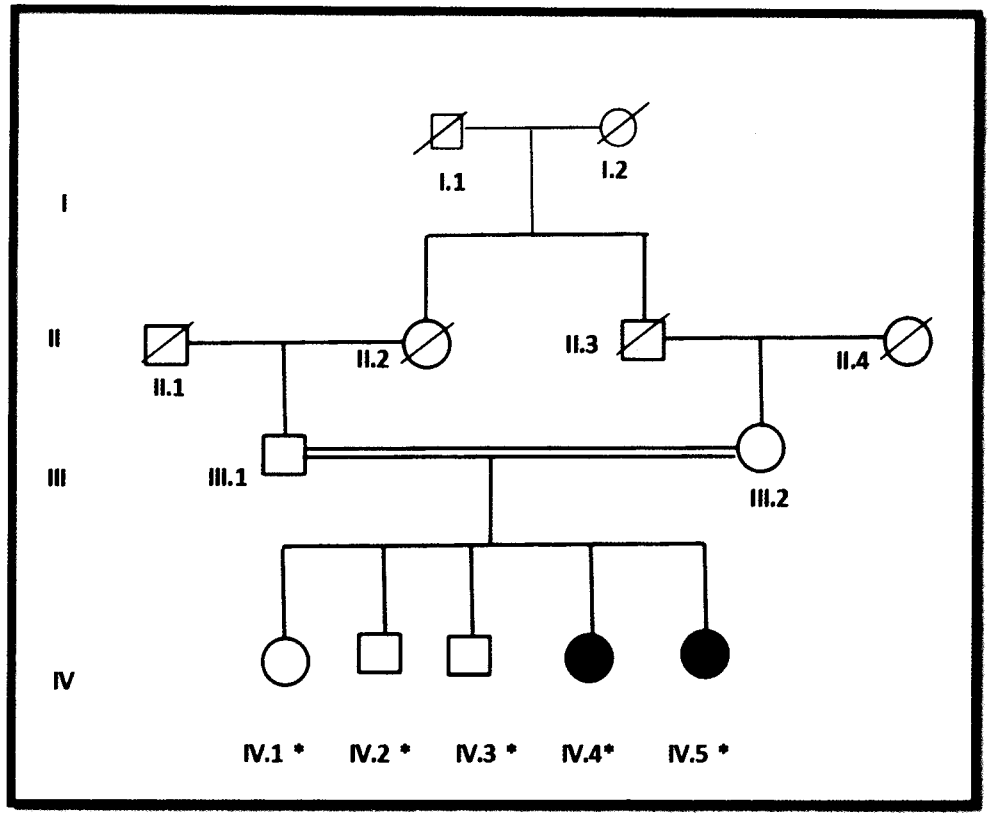


Figure 4.9 The family pedigree **(F)** which is clearly showing an affected individual III.5 and III.6. The II.1 (father), II.2(mother) both are normal and healthy. The mode of inheritance is autosomal recessive. (*) whichmeans to have blood samples of these family members.

Table 4.6 Initial Clinical Symptoms of Members of Family **(F)**

ID	Gender	Status	Age	Initial Clinical Findings		
				Consanguineous Parents	**Age onset**	**Location/Province**
II.1	Male	Normal	35years	Yes	N/A	Riwand/Punjab
II.2	Female	Normal	33years	Yes	N/A	Riwand/Punjab
III.4	Female	Affected	7years	Yes	By birth	Riwand/Punjab
III.5	Female	Affected	3years	Yes	By birth	Riwand/Punjab

Table 4.6.1 Detail clinical evaluation of Family **(F)**

Family F	Patient ID IV.4	Patient ID. IV.5
Gender	Female	Female
Age years	7 years	3 years
Status	Affected	Affected
regression started	At the age 4years	At the age 2years
Seizure	Yes	Yes
CNC	Encephalopathy	Encephalopathy
Vision	No	No
Memory loss	Yes	Yes
Speech	No speech	No speech
Behavioural abnormalities	Yes	Yes
EEG	Multifocal epileptiform discharges	Multifocal epileptiform discharges
MRI	Diffuse cerebellar and cerebralatrophy	Diffuse cerebellar andcerebral atrophy

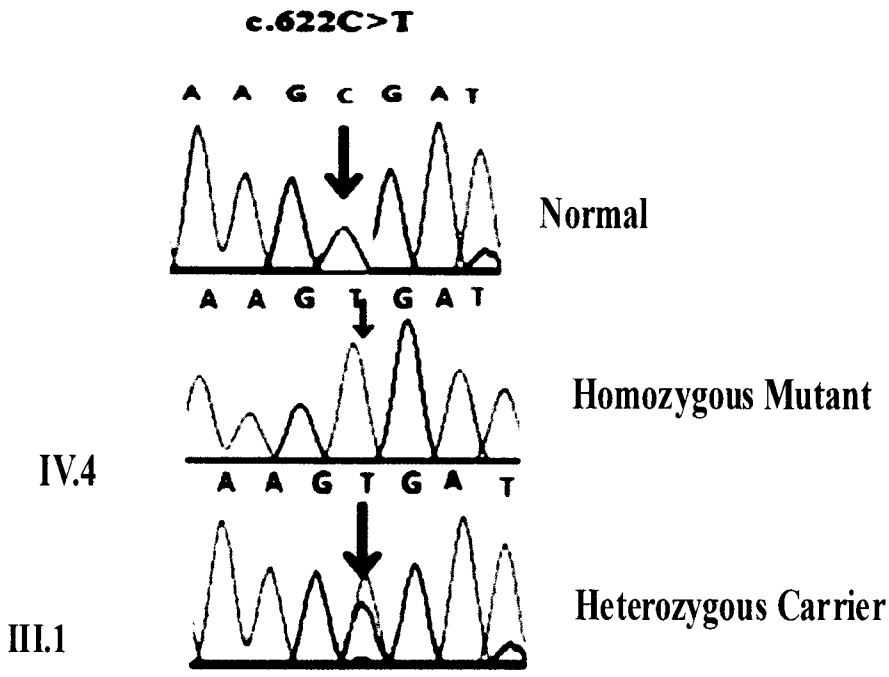


Figure 4.9.1 Variants and Sanger sequencing electropherograms confirming the variants in family **(F)**. IV.4 and IV5 are proband which is homozygous Mutant and III.1and III.2 is a healthy family member which is heterozygous carrier. in electropherograms clearly showing the mutation at the position c.622C>T.

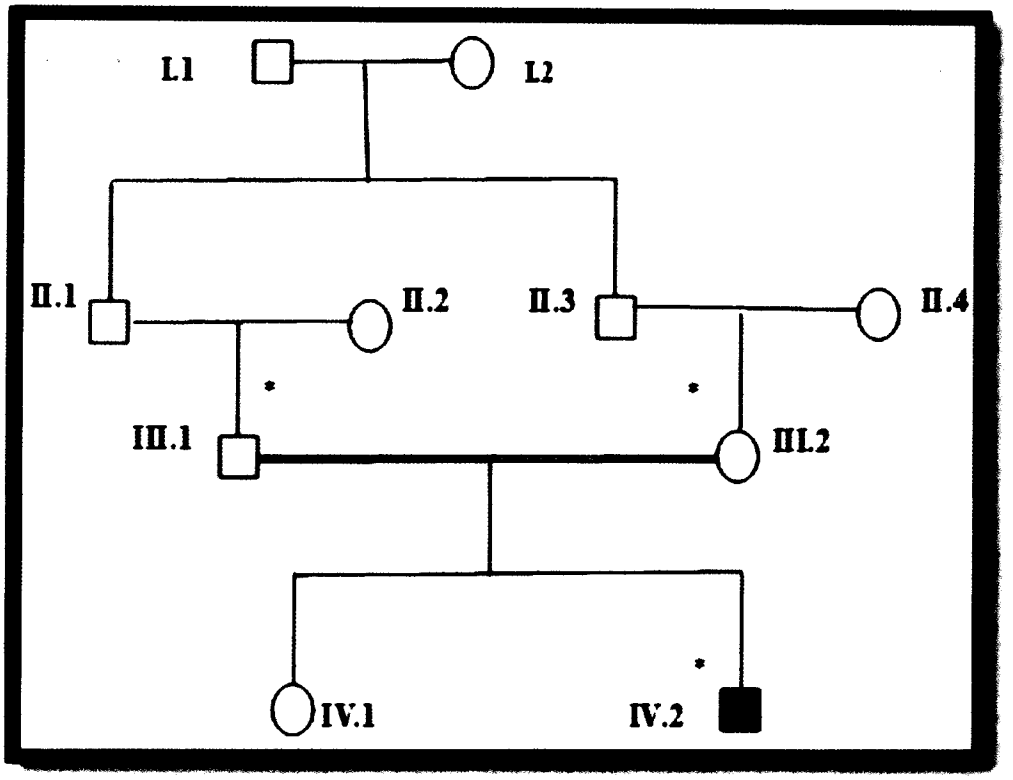


Figure 4.10 Family **(G)** which is clearly showing an affected individual IV.2. The III.1 (father), III.2(mother) and IV.1(sister) are normal and healthy family members. The mode of inheritanceis autosomal recessive. (*) which means to have blood samples of these family members.

Table 4.7 Initial Clinical Symptoms of Members of Family **(G)**

ID	Gender	Status	Age	Initial Clinical Findings		
				Consanguineous Parents	**Age onset**	**Location/Province**
III.1	Male	Normal	20years	Yes	N/A	Multan/Punjab
III.2	Female	Normal	22years	Yes	N/A	Multan/Punjab
IV.1	Female	Normal	9years	Yes	N/A	Multan/Punjab
IV.2	Male	Affected	7years	Yes	By birth	Multan/Punjab

Table 4.7.1 Detail clinical evaluation of Family **(G)**

Family G	Patient ID (IV.2)
Gender	**Male**
Age years	7 years
Status	Affected
Cerebral atrophy	Yes
regression started	At the age 4 years
Leukodystrophy	Yes
Intellectual disability	Mild
CNC	Encephalopathy
Speech	No
MRI	Deep white matter

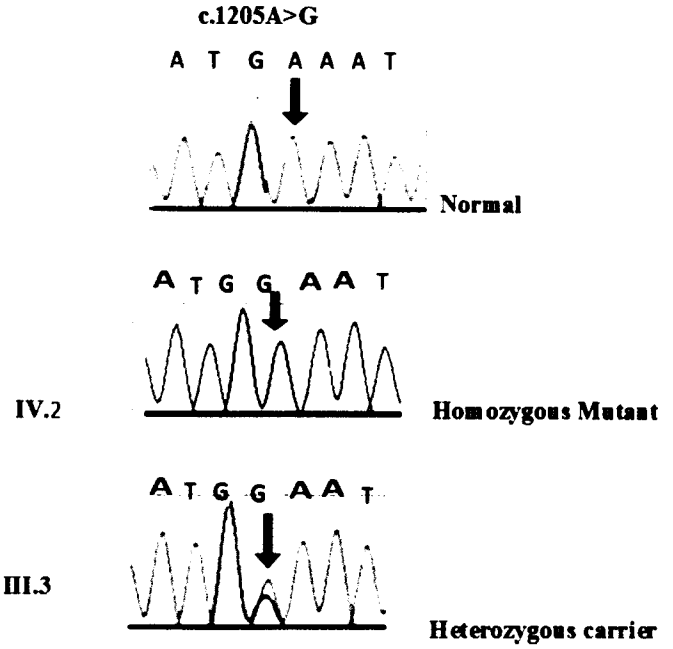


Figure 4.10.1 In family (**G**) the electropherograms clearly showing the alteration at the position c.1205A>G. IV.2 IS proband which is homozygous Mutant and III.1 is a healthy family member which is heterozygous carrier.

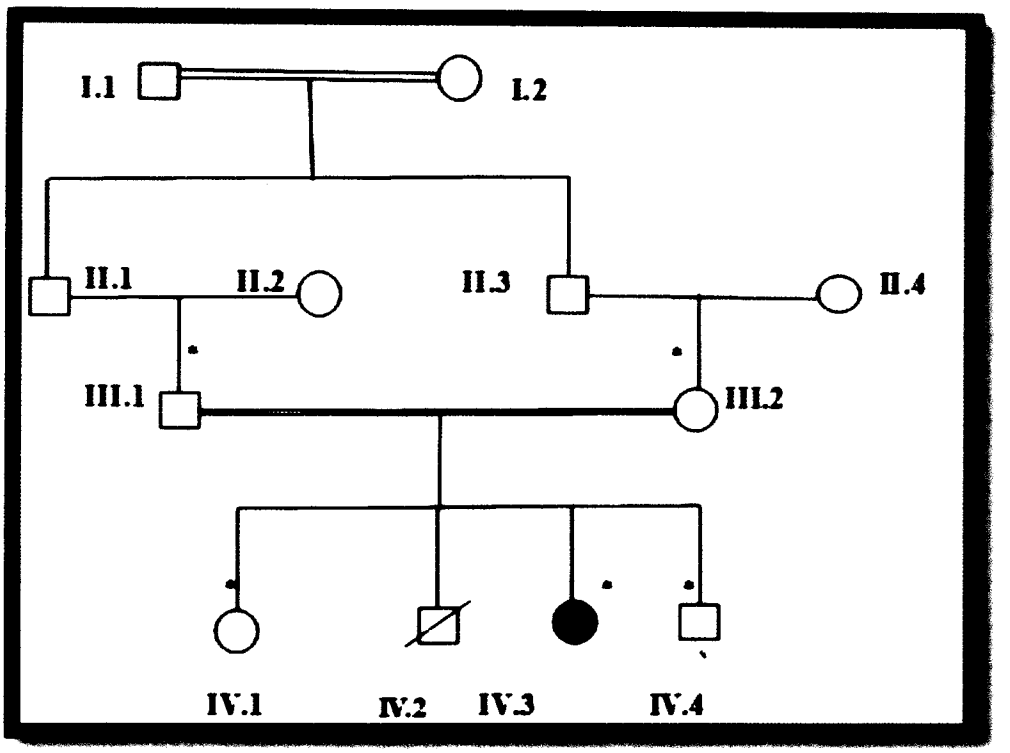


Figure 4.11 Family **(H)** which is clearly showing an affected individual IV.3. The III.1 (father), III.2(mother) and IV.1(sister) IV.4 (brother) are normal and healthy family members. The mode of inheritance is autosomal recessive. (*) which means to have blood samples of these family members.

Table 4.8 Initial Clinical Symptoms of Members of Family **(H)**

ID	Gender	Status	Age	Initial Clinical Findings		
				Consanguineous Parents	**Age onset**	**Location/Province**
III.1	Male	Normal	30years	Yes	N/A	Jhelum/Punjab
III.2	Female	Normal	25years	Yes	N/A	Jhelum/Punjab
IV.1	Female	Normal	10 years	Yes	N/A	Jhelum/Punjab
IV.3	Female	Affected	4years	Yes	By birth	Jhelum/Punjab
IV.4	Male	Normal	3years	Yes	N/A	Jhelum/Punjab

Table 4.8.1 Detail clinical evaluation of Family **(H)**

Family H	Patient ID IV.3
Gender	Female
Age years	4 years
Status	Affected
Cerebral atrophy	Yes
Regression started	At the age 2years
Intellectual disability	Yes
Seizure	Yes
CNC	Encephalopathy
Speech	No speech
EEG	Diffuse activity
MRI	Mild cerebella follicle

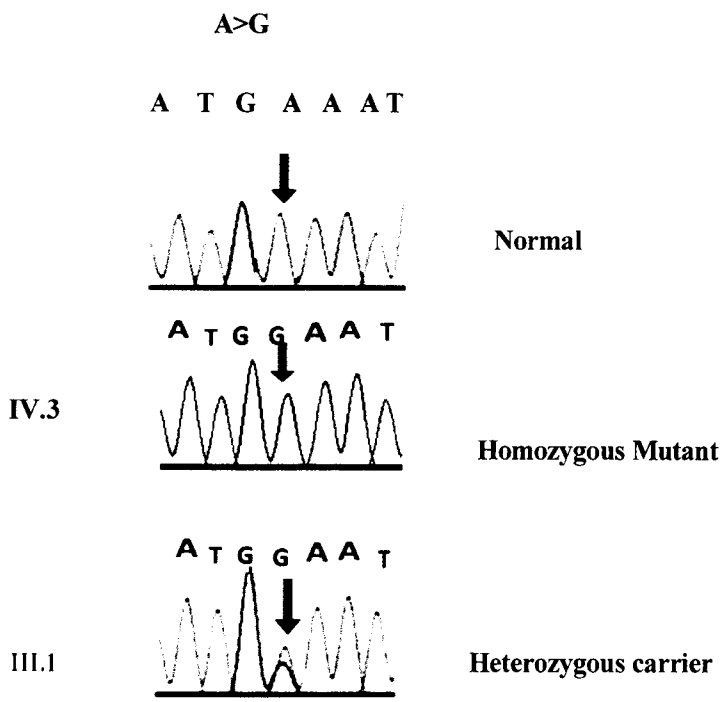


Figure 4.11.1 In family **(H)** the electropherograms showing the variants in which IV.3 is a proband which is homozygous Mutant and III.1 is a healthy family member which is heterozygous carrier. in electropherograms clearly showing the alteration at the position c.278A>G.

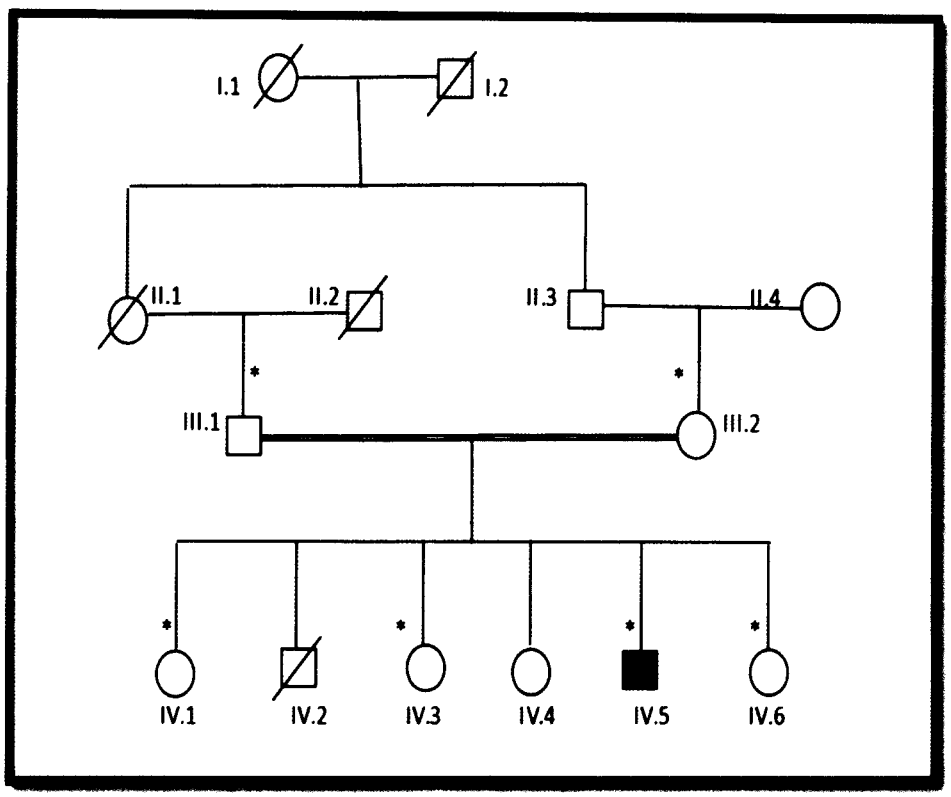


Figure 4.12 Detailed family pedigree of Family **(I)** which is evidently showing the mode of autosomal recessive inheritance.III.1 (father), III.2(mother), IV.1(sister), IV.3(sister) IV6(sister) all these are healthy family members.IV.5 is an affected individual. (*) which means to have blood samples.

Table 4.9 Initial clinical symptoms of members of Family **(I)**

Family ID	III.1	III.2	IV.5
Gender	Male	Female	Female
Location/Provence	Punjab	Punjab	Punjab
Age	55 years	60years	24 years
Status	Normal	Normal	Affected
Disease Appeared	No	No	12 years
Consanguineous Parents	Yes	Yes	Yes
Walk	Normal	Normal	Normal
Speech	Normal	Normal	Improper
Vision	Normal	Normal	Normal
Seizures	Normal	Normal	Yes
Intellectual disability	No	No	Mild

Table 4.9.1 Clinical Evaluation of Family **(I)**

Family I	Patient ID IV.5
Gender	Male
Age years	24 years
Status	Affected
Cerebral atrophy	Yes
Ataxic gait	Present
Dysarthria	Present
Speech	Improper
MRI	Normal

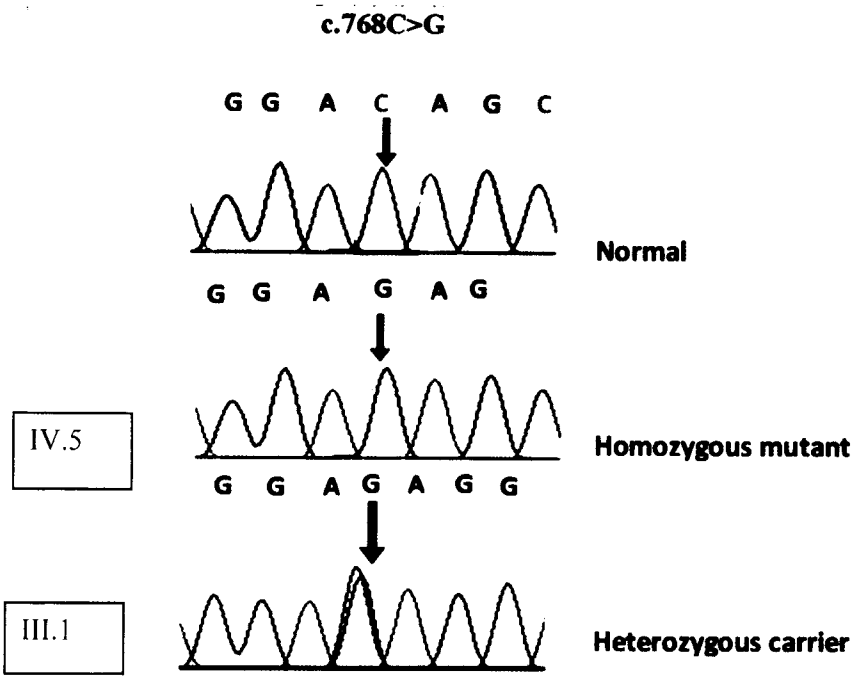


Figure 4.12.1 Variants and Sanger sequencing electropherograms confirming the variants in the families **(I).** IV.5 is a proband which is homozygous Mutant and I is a healthy family member which is heterozygous carrier. The peaks in electropherograms are clearly showingthe alteration at the position 768C>G.

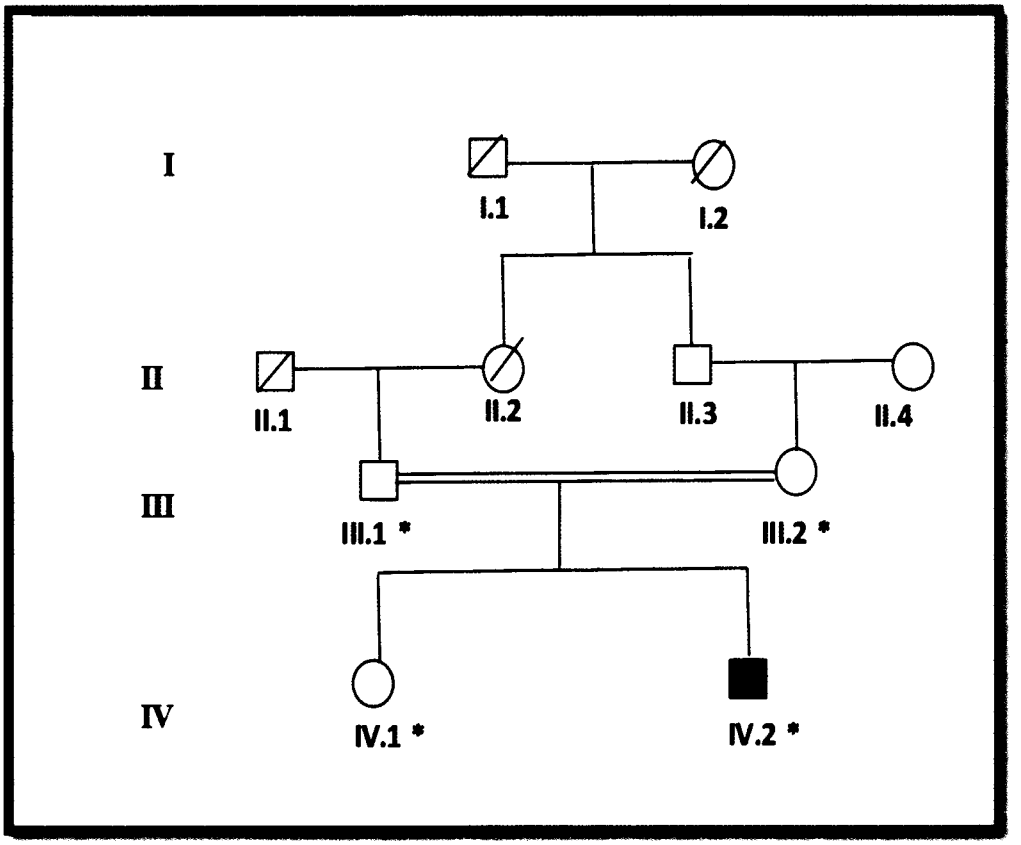


Figure 4.13 Detailed pedigree of Family-**J** which is clearly showing an affected individual IV.2. The III.1 (father), III.2(mother) and IV.1(sister) are normal and healthy family members. The mode of inheritance is autosomal recessive. (*) which means to have blood samples.

Table 4.10 Initial Clinical Symptoms of Members of Family **(J)**

Family ID	III.1	III.2	IV.1	IV..2
Gender	Male	Female	Female	**Male**
Location/Provence	Sahiwal/Punjab	Sahiwal/Punjab	Sahiwal/Punjab	Sahiwal/Punjab
Age	38 years	35 years	8 years	5 years
Status	Normal	Normal	Normal	Affected
Consanguineous Parents	Yes	Yes	Yes	Yes
Walk	Normal	Normal	Normal	Normal
Speech	Normal	Normal	Normal	Limited to 1-2 words
Regression of Mile Stones	No	No	No	Yes

Table 4.10.1. Clinical Evaluation of Family **(J)**

Family J	Patient ID (IV.2)
Gender	Female
Age years	5 years
Status	Affected
Psychomotor regression	Yes
Speech	Limited
Ophthalmologic	Bilateral optic disc pallor
Ataxia	Yes
Cerebellar atrophy	Yes
Gait difficulties	Severe
MRI	Diffuse cerebellar and cerebral atrophy

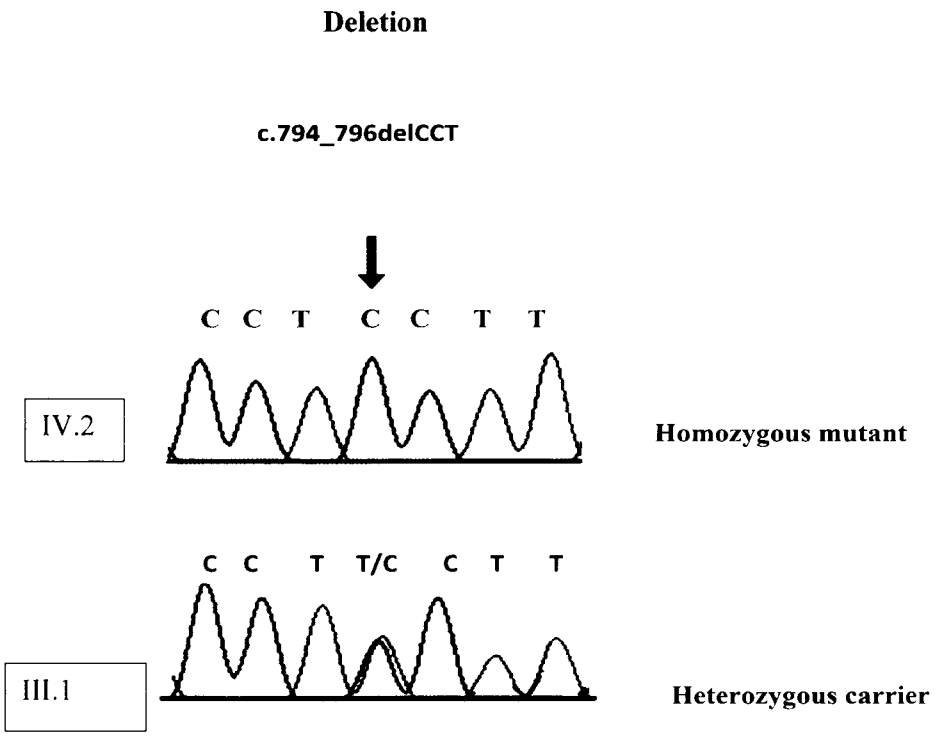


Figure 4.13.1 Variants and Sanger sequencing electropherograms confirming the variants in the families **(J)**. Co-segregation analysis proved that an affected individual (IV.2) is homozygous mutant for mutation p. Ser265del, c.794_796delCCT unaffected (III.1) is heterozygous carrier.

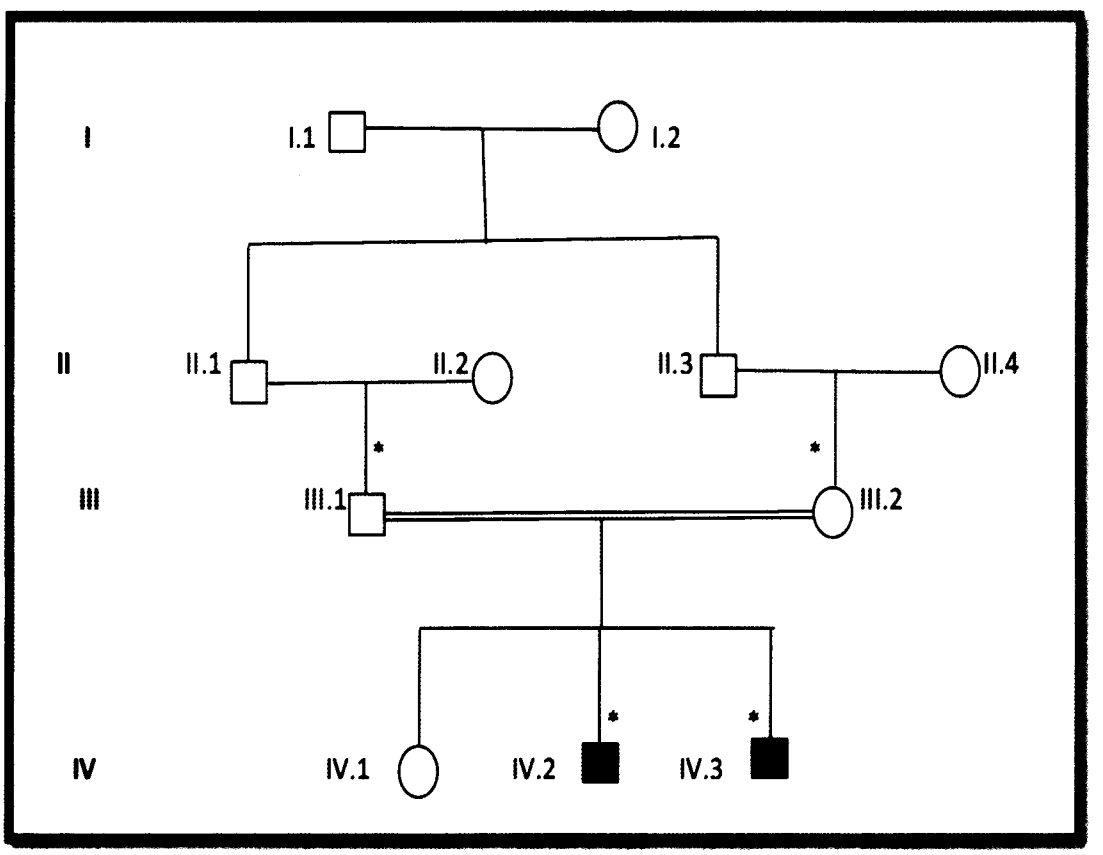


Figure 4.14 Detailed pedigree of Family **(K)** which is clearly showing an affected individual IV.2and IV.3. The III.1 (father), III.2(mother) and IV.1(sister) are normal and healthy family members. The mode of inheritance is autosomal recessive. (*) which means to have blood samples.

Table 4.11. Initial Clinical Symptoms of Members of Family **(K)**

Family ID. (K)	III.1	III.2	IV.1	IV.2	IV. 3
Gender	Male	Female	Female	Male	Male
Location/Provence	Punjab	Punjab	Punjab	Punjab	Punjab
Age	40 years	35 years	3 years	6 years	8 years
Status	Normal	Normal	Normal	Affected	Affected
Consanguineous Parents	Yes	Yes	Yes	Yes	Yes
Walk	Normal	Normal	Normal	Normal	Normal
Speech	Normal	Normal	Normal	Limited to 1-2 words	Less
Regression of Mile Stones	No	No	No	Yes	Yes

Table 4.11.1 Clinical Evaluation of Family **(K)**

Family K	Patient ID (IV.2)	Patient ID (IV.3)
Gender	Male	Male
Age years	6 years	8 years
Status	Affected	Affected
Psychomotor regression	Yes	Yes
Speech	Very limited	Less
Ophthalmologic	Bilateral optic disc pallor	Bilateral optic disc pallor
cerebellar atrophy	Yes	Yes
Seizures	Yes	Yes
EEG	Diffuse encephalopathy	Diffuse encephalopathy
MRI	Diffuse cerebellar and cerebralatrophy	Prominent cerebellar folia

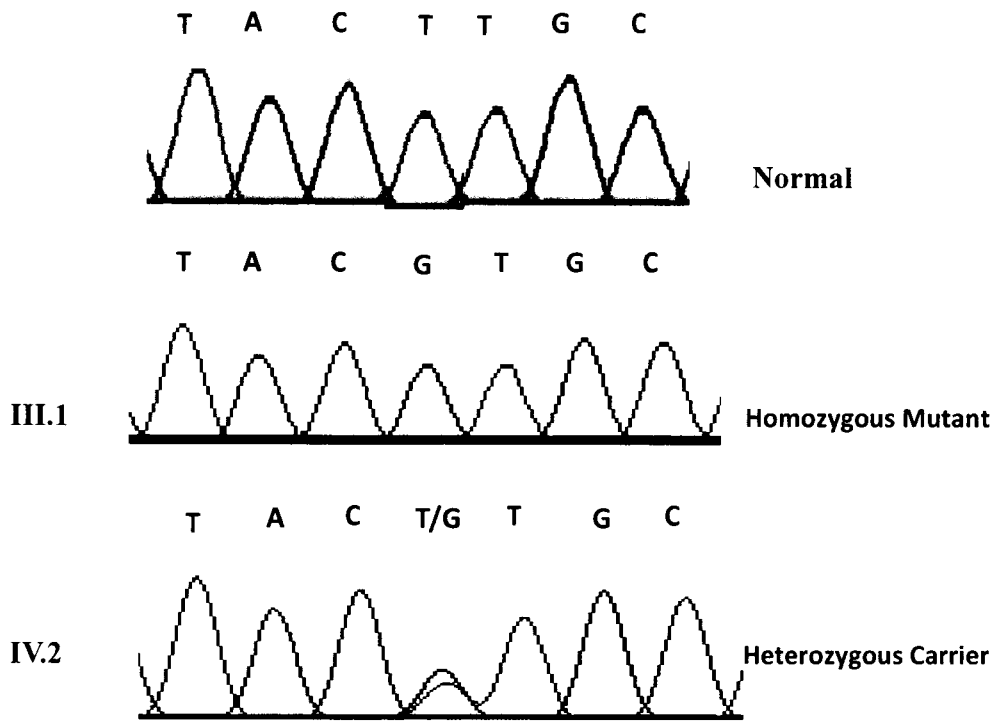


Figure 4.14.1 Variants and Sanger sequencing electropherograms confirming the variants in the families **(K).** Co-segregation analysis proved that an affected individual (IV.2 and IV.3) are homozygous mutant for mutation c.938T>G p. Leu313Arg unaffected (III.1) is heterozygous carrier. Electropherograms of whole family are given in appendix.

4.13 Results of Homology Modelling

In CLN5, template structure was covering 99% of the query sequence in which 97.6% sequence identity was present. While in *MFSD8*, multi template model was built using three templates to ensure reliable structure modelling. Selected models were having their DOPE score lower than 0 which indicate these models are at their lower stable state. GA341 score of selected models was greater than 0.7 which indicates the conservation of native folding in the structure. Ramachandran analysis done through MolProbity showed than human CLN5 model and human MFSD8 protein model has 98.144% and 99.13% residues in the allowed region, respectively, whereas, 95.51% and 96.43% residues were in the favored region of CLN5 model and MFSD8 models, respectively. Graphical representation of the models was prepared using Maestro shown in figure 14.16 and figure14.18.

4.14 Prediction of Protein stability changes

The Blundell lab has developed multiple mutation analysis tools, which estimate the impact of alterations on protein structure constancy using a machine-learning tactic based on graph-based cumulative distance signatures and statistical potential energy functions. This stability change in proteins is quantified in terms of the change in the Gibbs free energy ($\Delta\Delta G$), which further helps to elucidate the structural and functional impacts responsible for the disease phenotype. Thermodynamic changes and/or effects of mutations on human CLN5 and human MFSD8 was analyzed by investigating impacts of mutations stability in protein (mCSM-PS), protein-protein interaction (mCSM-PPI), protein-nucleic acid (mCSM-DNA) and protein-metal (mCSM-NA)and SDM.

SDM predicted secondary structure switches in mutants, change in relative side-chain solvent accessibility (RSA), depth of the residue in Å and residue-occluded packing densities were considered in mutation analysis. Mutant models will be generated using Andante (Smith *et al.*, 2007), a tool integrated with SDM. To improve the overall accuracy of the mutation prediction, DUET analysis was performed which integrates both the approaches of MCSM and SDM

Table 4.12 Parameters of homology modelling and structure quality analysis of *CLN5* and *MFSD8*.

Gene	Organism	Amino Acid	Template	Query Coverage	Sequence identity	DOPE	GA341>0.7	Ramachandran Analysis	
								Allowed Region	Favored Region
CLN5	Homo Sapiens	358aa	6R99_A	99%	97.6%	-0.82	1	98.44%	95.51%
MFSD8	Homo Sapiens	518aa	1PW1_A 6E90_A 3WDO_A	63%,70% 22%	55%,25%, 33.61%	-1.55	1	99.13%	96.43%

Table 4.13 List of experimentally known mutations and their structure-driven mutation analysis.

Mutation	MCSM-PS	MCSM-PPI	SDM	DUT
		CLN5		
C159R	-1.84 kcal/mol	-0.553 kcal/mol	-0.3kcal/mol	-1.59kcal/mol
L309A	-1.43kcal/mol	0.68kcal/mol	-0.9kcal/mol	-1.46kcal/mol
		MFSD8		
L313R	-1.46	-1.064kcal/mol	-2.78Kcal/mol	-1.53kcal/mol

*** Energy value ΔΔG<0kcal/mol: Destabilizing mutations**

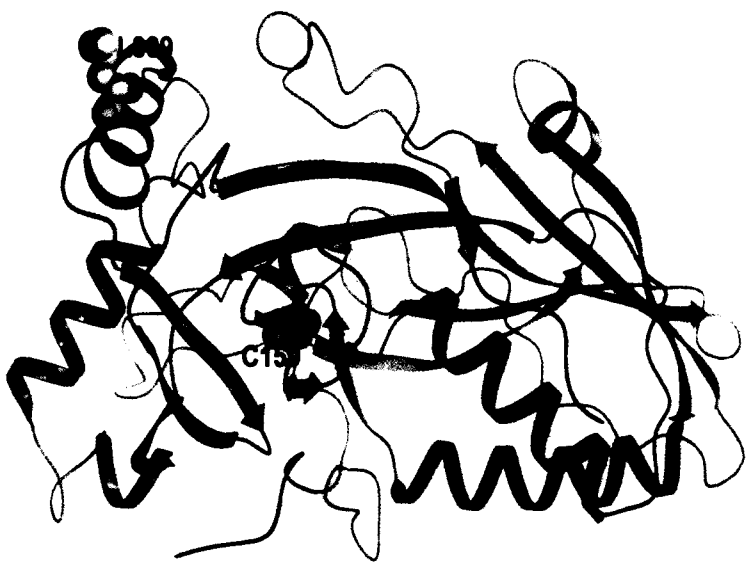


Figure 4.15: The normal protein structure at position,159 and another: c.925_926del in *CLN5* gene.

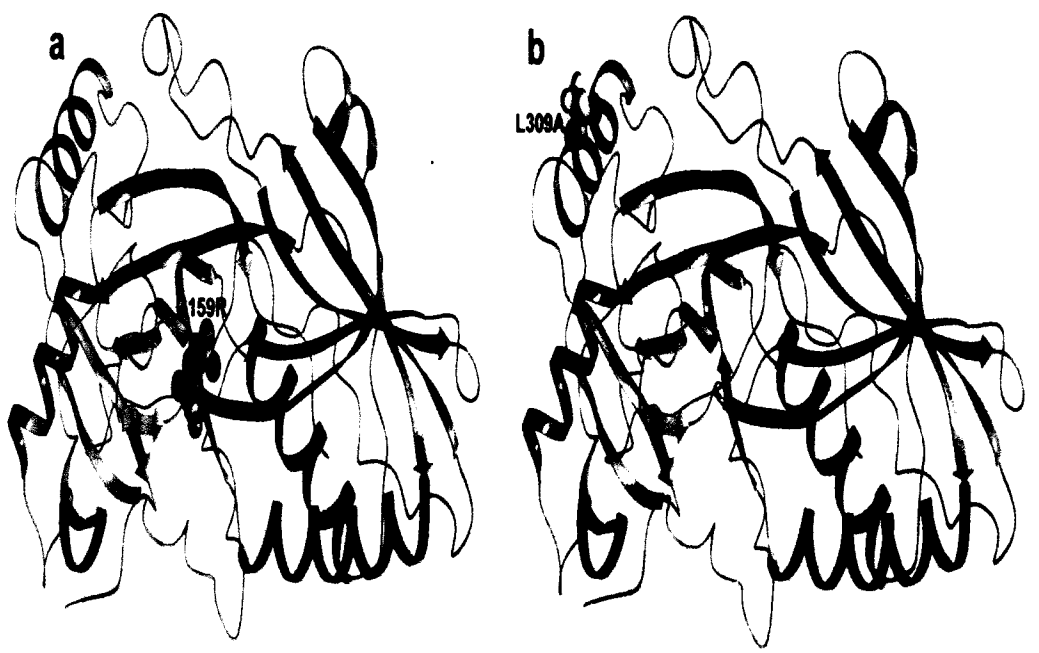


Figure 4.16: Mutant structure of protein at position number 159 with change p. Cys159Arg and c.925_926delwithin *CLN5* gene.

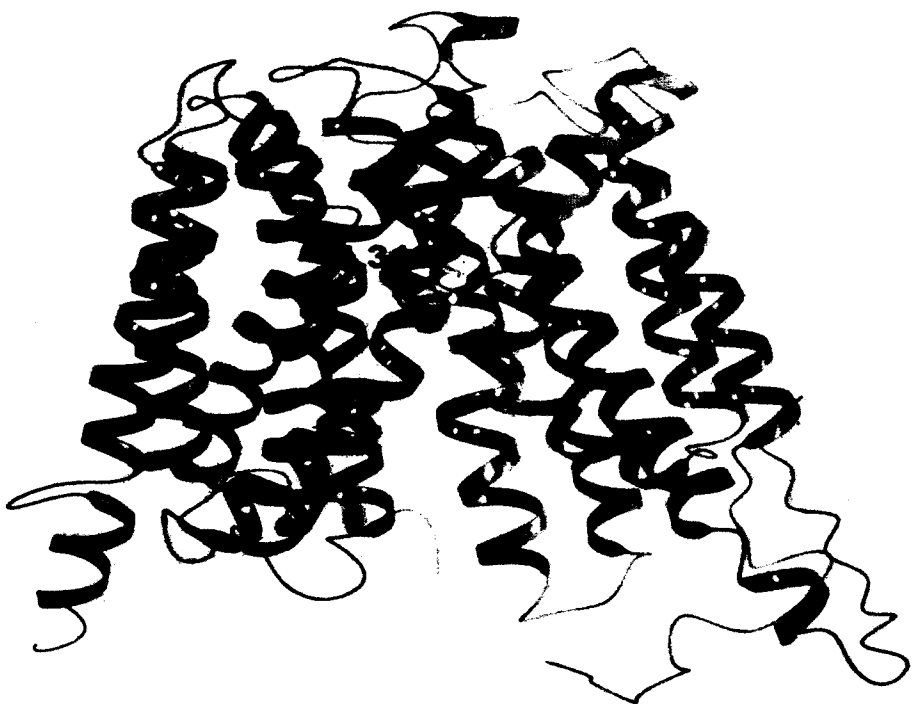


Figure 4.17: The normal protein structure at position,313, c.925_926del in *MFSD8* gene.

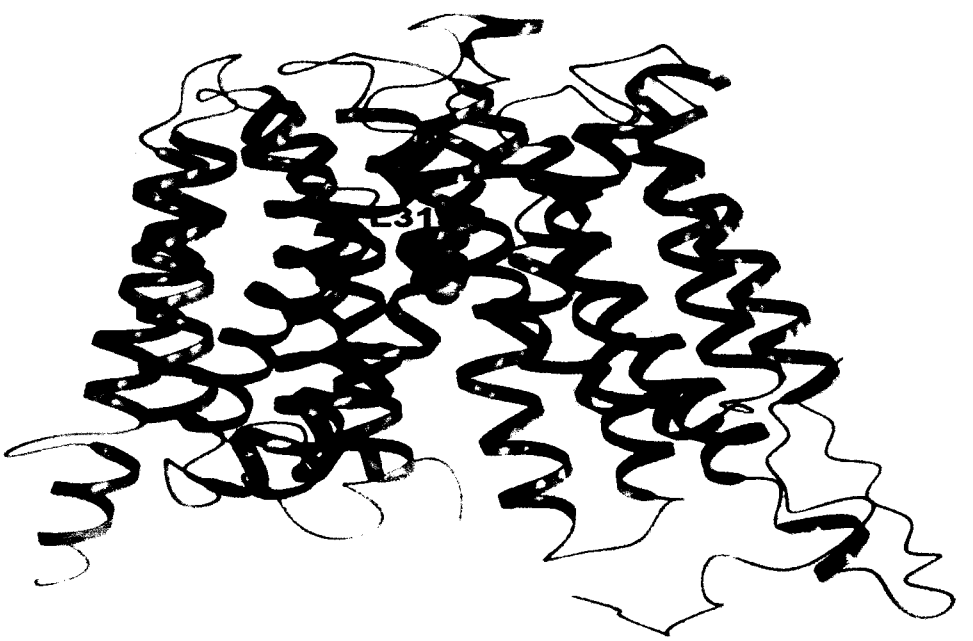


Figure 4.18: Mutant structure of protein at position number 313 with change p. Leu 313Arg homozygous missense variant NM_152778.2, c.938T>G(chr4:12,88,51,898) in *MFSD8* gene

5 Discussions

Genetic research provides the chance to learn more about the causes of inherited disorders. Once the underlying genetic cause of a disorder has been identified, diagnostic test may be carried out to give affected families the opportunity for early diagnosis and treatment intervention. Neurodevelopmental disorders (NDDs) are a group of diseases that impact how the brain develops and functions. They are identified by a wide degree of genetic and clinical variability (Parenti, *et al.,* 2020). The Neuronal Ceroid Lipofuscinoses are the childhood onset neurodegenerative disorder which is mostly affecting brain and retina (Simonati and Williams., 2022). Neuronal Ceroid Lipofuscinoses is known as a lysosomal storage disease, affected by more than 530 mutations in the NCL gene (Koohmanaee *et al.*, 2023). Batten and Vogt both described the first instance of childhood-onset NCL in 1903 and 1905, respectively (Koohmanaee, *et al.*, 2023) but Stengel made the initial diagnosis in 1826(Al-Muhaizea *et al.*, 2009). The classification of NCLs is depend on the defective protein or gene as well as the age of onset clinical symptoms (Baranzehi *et al.*,2022). NCL is classified into four main groups including infantile (Haltia-Santavuori disease), late in- fantile (Jansky-Bielschowsky disease), juvenile (Batten- Spielmeyer-Vogt disease), and adult types (Kufs Parry disease) (Koohmanaee, *et al.*, 2023).

NCLs are categorized into 4 main subtypes which include infantile (INCL), later infantile (LINCL), juvenile (JNCL) and adult (ANCL) (Parvin *et al.*, 2019). The onset of loss of vision leading to blindness, degeneration in cognitive and motor capabilities, and epileptic seizures in early young age may underlay the change into NCLs. The beginning of such symptoms varies in accordance to the type of NCL in each individual (Hemanth *et al.*, 2020). CLN5 disease was initially identified as an infrequent variant (Savukoski *et al.,* 1998). The NCL-linked proteins exhibit great variation in cellular localization despite the fact that they all regulate the lysosomal degradation process.n (Hughes *et al.,* 2014). Recent evidences have shown that CLN5 may also localize extracellularly (Huber and Mathavarajah., 2018). The most of NCL-associated proteins have been reported to occur in lysosomes (CLN1, CLN2, CLN3, CLN5, CLN7, CLN10, CLN12, and CLN13), but they have also been discovered in the cytosol (CLN6 and CLN8) and endoplasmic reticulum (CLN6 and CLN8) in association with vesicular membranes (CLN4 and CLN14) (Carcel *et al.*,2015).

In few case reports, patients have been reported to have many types of combined mutations (Kousi *et al.*,2012). There are many different types of diseases, each with a unique course, severity, and age of onset. It is important to keep in mind that a gene mutation's type does not always indicate a disease's phenotype, and vice versa. The age of NCL onset is not proportional to the severity of the malfunction caused by the mutant gene (Gardner and Mole 2021). To diagnose the type of disease some findings may help. CLN3 is linked with vacuolated lymphocytes in blood smears which are pathognomonic for this type (Willemijn *et al.*,2020). Contrary to all other forms, which are inherited in an autosomal recessive fashion, CLN4 (Kufs Parry disease) is inherited in an autosomal dominant manner. Although it is characterised by adult-onset presentation, recessive mutations can occasionally cause late-onset. (Naseri *et al.*, 2021). Kufs disease typically manifests as dementia after the age of 30, with vision remaining intact (Koohmanaee *et al.*,2023). Except for those who have a rare congenital variant (CLN10), all NCL patients appear to have normal psychomotor development prior to the onset of major symptoms (Samuel *et al.*, 2019). In 2019, two NCL cases were documented, both with typical Batten disease symptoms such as seizures, developmental regression, ataxia, dysarthria, dysphagia, dystonia, and impaired cognitive function, but without visual impairment (Joseph *et al.*,2019). CLN6 exhibited its first symptoms during the late infantile period, according to a previous study; however, CLN6 mutation is the most common cause of type A (autosomal recessive) Kufs disease (Berkovic *et al.*,2019). CLN6, which has over 60 known mutations and is located on 15q21-q23, was first identified in Costa Rican and Venezuelan patients. Missense and nonsense mutations, small deletions or insertions, and splice-site mutations have all been reported thus far. (Koohmanaee, *et al.*, 2023).

Our study comprised of eleven families. The main purpose of the conducted study was to identify the causative gene associated to NCL. Different genes that belong to various families were included. Whole exome sequencing was carried out on respective families and segregation analysis was done by Sanger sequencing. We collected the blood samples of the families from different areas of Pakistan. The blood samples were collected for the affected as well as normal members of each family. We labelled the respective families from A to K alphabetically. Family A belonged to Khyber Agency, Pakistan. We identified CLN5 gene mutation in Family A. Family B belonged to Gujrat region of Punjab and it had also reported novel mutation on CLN5 gene This was a novel mutation which was never reported before.

We discovered two novel homozygous CLN5 variants in these two families: the c.477 T > C, p. Cys159Arg, and the c.925 926del, p. Leu309AlafsTer4. Our patients are also the second CLN5 cases in the Pakistani community, following the 2009 report of the country's first Asian family. Up-to this time, most cases of CLN5 disease have been described in Finnish and northern European nations just as in Italy and China. Our discoveries demonstrated that similar CLN5 pathogenic variations are similarly present in the Punjab territory, resulting is a more worldwide distribution of CLN5 disease. (Lebrun *et al.*, 2009). Family C was from Haripur, we found the reported *CLN5* stop gained variant c.524T>A, p. Leu175Ter (chr13: 77,570,074). We worked on five families (D, E, F, G, H) which were belonging to different regions of Pakistan as mentioned before in results section, these families were related to with *TPP1* gene.

We identified three *TPP1* variants which were first time reported in Pakistani families but before that were identified in different countries. Homozygous mutations in *TPP1* were identified with the homozygous missense variant c.616C>T, p. p. Arg206Cys (chr 11-66,38,277).in two families another homozygous mutation in *TPP1* were identified with the homozygous missense variant c.622C>T, p. Arg208Ter (chr11-66,38,271) in one family. In family D and E, the homozygous missense variant p. Glu402Gly c.1205A>G was identified. After proper examination by MRI findings of cerebellar atrophy involves neurodegeneration in the pathogenesis of the CLNs disease. In family G the homozygous missense variant p. Glu402Gly c.1205A>G was identified. Family H belonged to Jhelum, in which homozygous mutations in *TPP1* gene with mutation Ter93TrpextTer3: c.278A>G was identified. Homozygous mutations in CLN6 were identified in the family-I. In family I, the homozygous missense variant: c.768C>G, was identified Family J belonged to Sahiwal; Homozygous mutations were identified in this family. With deletion p. Ser265del, c.794_796delCCT Family K belongs to Faisalabad homozygous mutation with a novel homozygous alteration in MFSD8 gene. In this family, the novel homozygous missense variant c.938T>G p. Leu313Arg) was identified

My whole research highlights effect of CLN in Pakistani families. Next generation sequencing based studies involving populations from various ethnic backgrounds would assist to identify the particular global distribution of the rarest NCLs in the future, also contributing to the further explanation of the related clinical phenotypes.

The affected families would benefit from this evidence in terms of clinical management, prognosis, family development, and prenatal diagnostics. Families from various regions of Pakistan who had NCL were enrolled. After linkage analysis, exome sequencing, bioinformatic analysis, and the identification of structural changes in the mutant protein were revealed. Significant genetic knowledge about NCL was added to the body of knowledge during the course of this work. This will help future study by enabling it to clarify and understand the molecular pathophysiology of NCL.

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ANNEXURES

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Novel likely disease-causing *CLN5* variants identified in Pakistani patients with neuronal ceroid lipofuscinosis

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Exome sequencing

ABSTRACT

Background: Neuronal ceroid lipofuscinosis (NCL) is a hereditary lysosomal storage disease with progressive brain neurodegeneration. Mutations in ceroid lipofuscinosis neuronal protein 5 (CLN5) cause CLN5 disease, a severe condition characterized by seizures, visual failure, motor decline, and progressive cognitive deterioration. This study aimed to identify causative gene variants in Pakistani consanguineous families diagnosed with NCL.

Methods: After a thorough clinical and neuroradiological characterization, whole exome sequencing (WES) was performed in 3 patients from 2 unrelated families. Segregation analysis was subsequently performed through Sanger sequencing.

Analysis: WES led to the identification of the 2 novel homozygous variants c.925_926del, (p.Leu309AlafsTer4) and c.477 T > C, (p.Cys159Arg).

Conclusion: In this study, we report two novel *CLN5* cases in the Punjab region of Pakistan. Our observations will help clinicians observe and compare common and unique clinical features of NCL patients, further improving our current understanding of NCL.

1. Introduction

Neuronal ceroid lipofuscinoses (NCLs) are a group of rare inherited lysosomal storage disorders leading to fatal progressive neurodegeneration [1–4]. They affect every age and gender with a global distribution [5]. NCLs are one of the most frequent childhood-onset neurodegenerative conditions [6]. Common symptoms include seizures, progressive vision impairment, and decline in motor and cognitive functions. The diffuse involvement of the nervous system and the neurodegenerative course usually lead to premature death [7]. NCLs show a large clinical and genetic heterogeneity. To date, 13 different forms have been identified and classified according to the age of onset and affected gene [8].

Mutations in ceroid lipofuscinosis neuronal protein 5 (CLN5) cause CLN5 disease, a severe and rare form of NCL manifesting between 2 and 8 years of age, and therefore classified as late-infantile NCL (LINCL). The first symptom is usually cognitive decline with reduced learning ability often involving verbal functions. This is usually followed by seizures and visual impairment, as well as progressive decline in motor functions, usually starting with clumsiness [9–12]. Compared to other NCLs, seizures and vision impairment occur relatively late in the disease course and seizures progression is slower than early-infantile and infantile forms [13].

Here, we identified 2 novel homozygous variants in *CLN5* in 3 patients from 2 unrelated consanguineous Pakistani families identified as part of a consortium study.

2. Materials and methods

2.1. Identification of affected individuals and collection of samples

All families were collected as part of the SYNaPS Study Group collaboration funded by The Wellcome Trust, which looks at rare disease-causing variants in consanguineous families presented with

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Available online 07 April 2020

1.

C	08-04-2019

Date	08-10-2018
Family No.	
UCL Lab. No	
Mobile: I	0300-5582096
Mobile: II	0301-2469816
Landline:	
Address:	Khayber Agency
Booked By	Dr. Azeem

Naila(10yrs) & Hayat Wali (07 yrs)

Regression of Mile Stones: evident psychomotor regression

Speech / Hearing / Vision:

Neurological Assessment: naila regression started at the age of 7 years with behavioral changes and memory loss, followed by frequent falls evealed cerebellar sings (ataxia, dysmetria, and tremors).

Systemic Review

Metabolic work up: Urinary copper: 76µg, AFP: raised 5.24 IU/ml, LFT's, RFT's: normal.

MRI Lumber Spine: Focal posterior left herniation of L5-S1, Disc compression at left distal nerve roots.

Hayat behavioral abnormalities (episodes of laughter, agitation, and wandering), memory loss, and impaired night vision at 6 years of age

MRI Brain : (Naila) diffuse cerebral and cerebellar atrophy

EEG: multifocal epileptiform discharges. In both

Audiometry:

Ophthalmologist Notes: optic disc Pallor

EMG/NCS: Not done

Clinical Videos: Attached

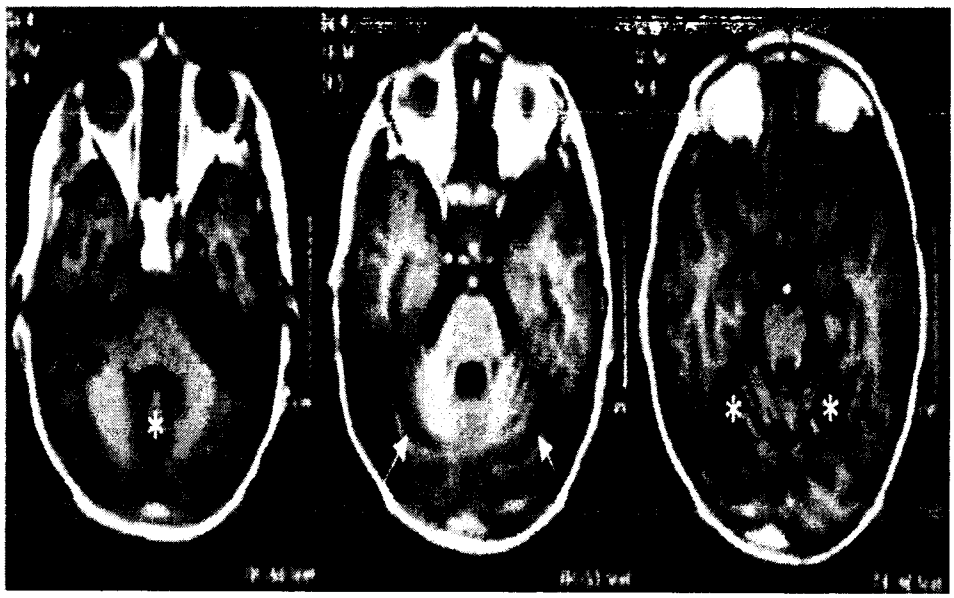
Diagnosis:

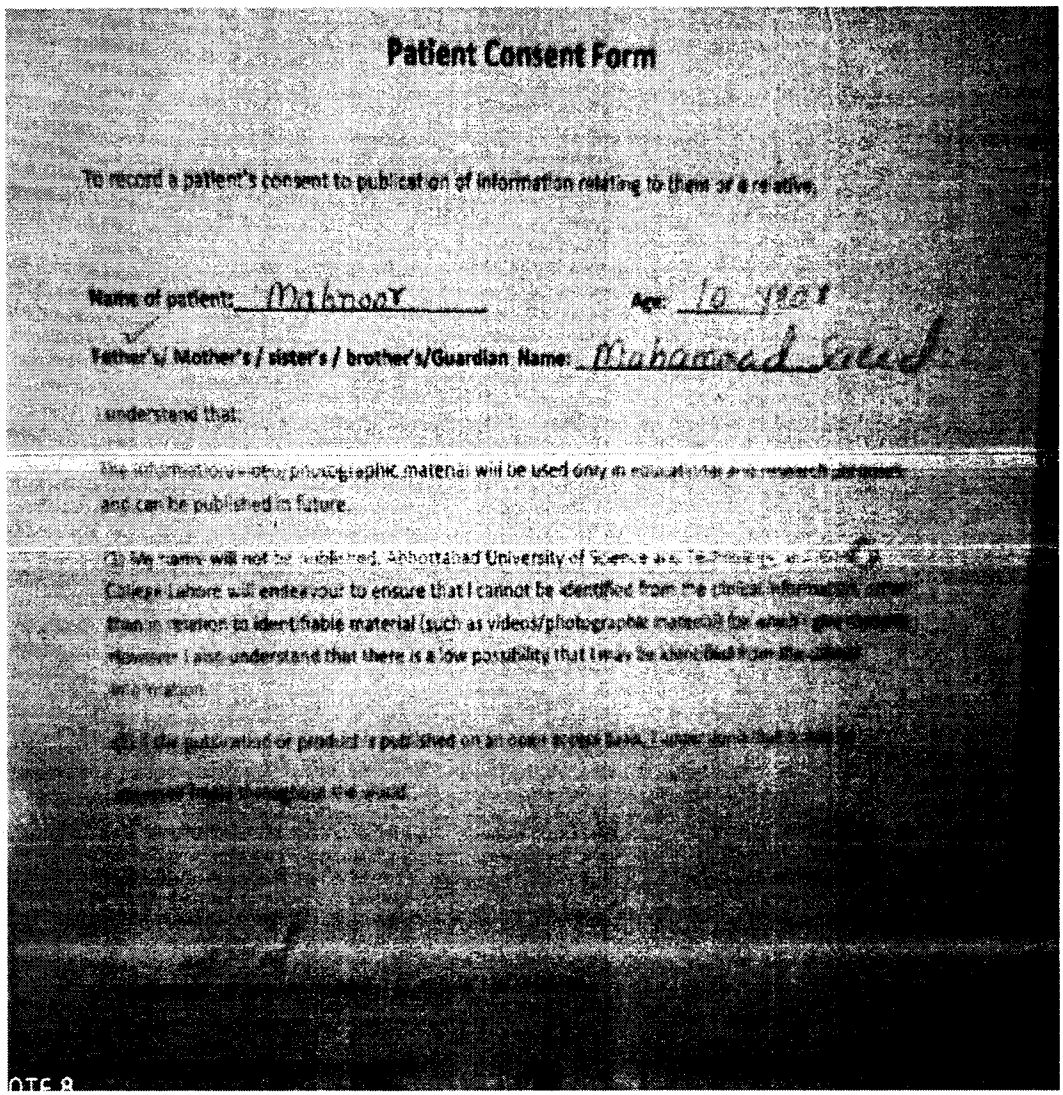
Family No.	H
uCL Lab. No	
Mobile: I	0333-8421878
Mobile: II	0313-3383007
Landline:	
Address:	Gujrat
Booked By	Prof. Tipu Sultan

Ahmed(08years)
Regression of Mile Stones: Deteriorating school performing followed by fits, (Drop attacks + Tonic clonic) with frequentfalls, then regression of walking, sitting over 2 years time period.
Speech / Hearing / Vision: Speech affected, Hearing Normal, Vision: Normal.
Neurological Assessment:OFC: 51cm, Excessive drooling during examination,Ataxic gait with positive cerebellar signs (dysmetria, incordination), Tone: decreased, Reflexes: ellicitable, Planters: Upgoing.
Systemic Review: Normal
Metabolic work up:SGPT: 17, RFTs: Normal, TSH: 9.9(0.4-5.0mIU/L), T3,T4: Normal.
MRI brain: Cerebellar atrophy.
EEG: Multifocal discharges.
Audiometry: Not done.
Ophthalmologist Notes: Normal fundus, No optic atrophy.
EMG/NCS: N/A
Clinical Videos: Attached
Diagnosis: Neurodegenerative disorder

2.

Neuroimaging of Family A and B





Patient Consent Form

To record a patient's consent to publication of information relating to them or a relative.

Name of patient: Mahnoor Age: 10 years

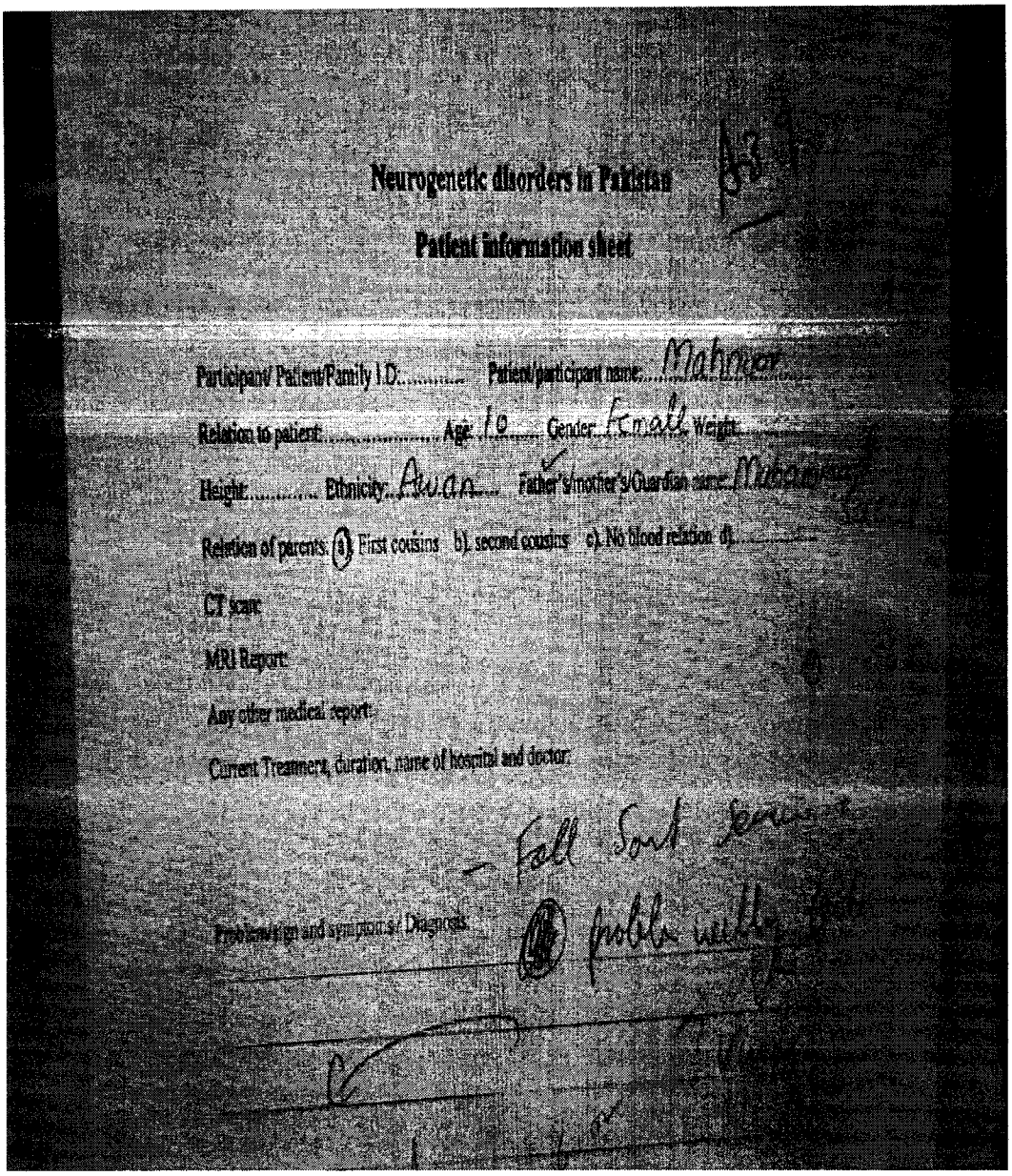
Father's/ Mother's / sister's / brother's/Guardian Name: Muhammad Saeed

I understand that:

The information or photographic material will be used only in publications in peer-reviewed research journals and can be published in future.

(2) My name will not be published. Abbottabad University of Science and Technology and Shifa College Lahore will endeavour to ensure that I cannot be identified from the clinical information unless there is mention to identifiable material (such as videos/photographic material) for which give consent. However I also understand that there is a low possibility that I may be identified from the clinical information.

(3) If the publication or product is published on an open access basis, I understand that it may be accessed from throughout the world.



Neurogenetic disorders in Pakistan

Patient information sheet

Participant/ Patient/Family I.D:..... Patient/participant name:.....

Relation to patient:..... Age:..... Gender:..... Weight:.....

Height:..... Ethnicity:..... Father's/mother's/Guardian name:.....

Relation of parents: a). First cousins b). second cousins c). No blood relation d).....

CT scan:

MRI Report:

Any other medical report:

Current Treatment, duration, name of hospital and doctor:

Problem/sign and symptoms/ Diagnosis:

Fajar (05 years)

Regression of Mile Stones: At 3 years of age epilepsy: Motor, Regression: Globalregression, decrease cognition

Speech / Hearing / Vision: Hearing? , Vision: Normal, Speech: Affected

Neurological Assessment: Tone: Increased, Power:4-5/5, Reflexes: Increased, Planters:Up going.

Systemic

Review:N.A

Metabolic work

up:N.A

MRI Brain: Cerebral atrophy with foci of demyelination in periventricular white matter.

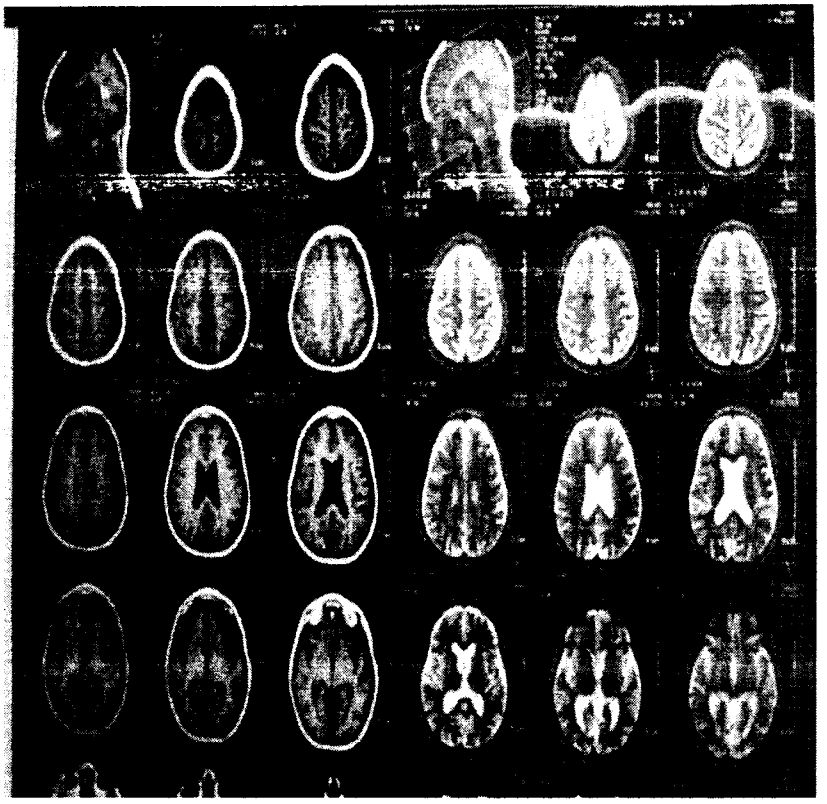
EEG: Multifocal epileptiform activity

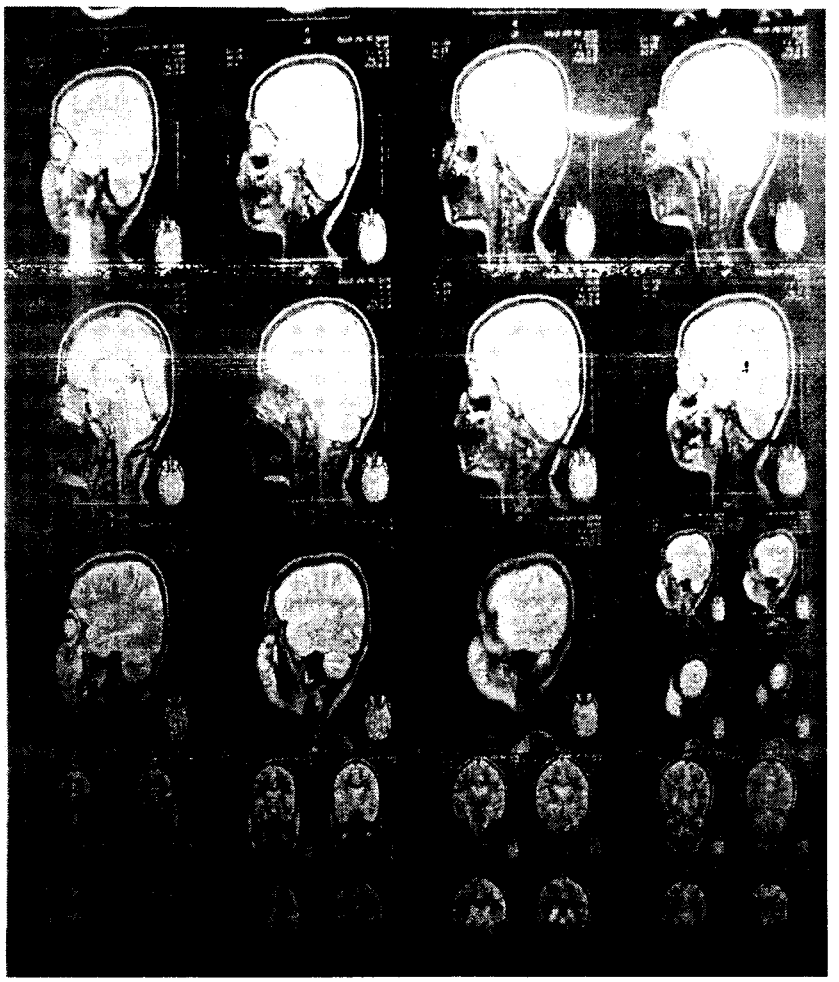
Audiometry:N.A

Ophthalmologist Notes: Fix and follow light**EMG/NCS AND Clinical**

Videos: Attached Diagnosis: DBD

Family c





THE CHILDREN'S HOSPITAL & THE INSTITUTE OF CHILD HEALTH
[illegible] ROAD, LAHORE (PAKISTAN)

DIAGNOSTIC RADIOLOGY DEPARTMENT

Ref: 1131

Date: 17-02-2016

PATIENT NAME: Fajar Shahid	AGE/SEX: 4.6 Years/Male
AREA SCANNED: Brain	REF BY:

MRI REPORT

Clinical [illegible]:

OBSERVATION :-

Multi-[illegible] images through the brain were performed.

Ventricular and extra ventricular CSF spaces are dilated.

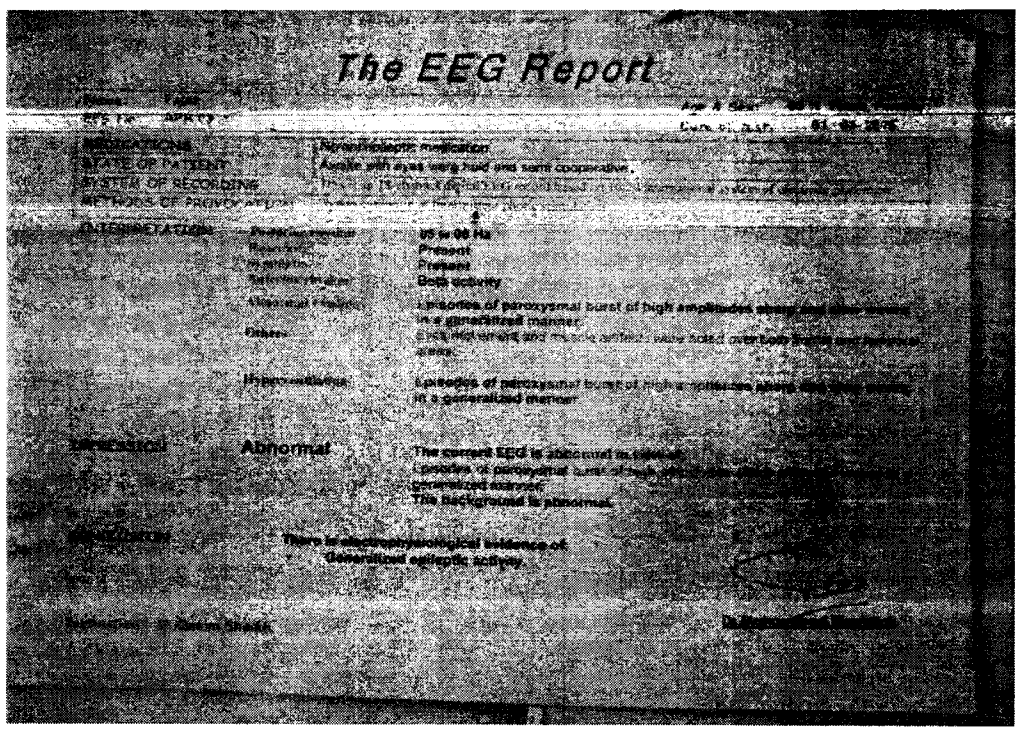
Subtle [illegible] hyperintensities seen along centrum semiovale and peri-ventricular white matter demyelination.

Loss of flow void in right transverse sinus can be due to slow flow.

There is no evidence of recent or remote infarct. Brainstem and both cerebellar hemispheres appear normal. Both hippocampi appear normal & symmetrical. Pituitary gland and optic chiasm are normal. 7th & 8th nerve complexes are normal & symmetrical bilaterally. Cranio-vertebral junction appears normal. No evidence of sino-sinus or oto-mastoid inflammation.

CONCLUSION :-

- **MRI features are of cerebral atrophy with focci of demyelination in periventricular white mater.**


The EEG Report
Abnormal

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Name: S/O Mr Jahaazaib	ID No.	Age: Yrs	Sex: M/F
Unit: ---	Ward: ---	Date: 7-2-2021	

MRI BRAIN

CLINICAL DATA

- Cerebellar ataxia, improper speech

EXAMINATION TECHNIQUE

- Multi-planar imaging done through brain acquiring T1/T2 weighted and FLAIR sequences.

FINDINGS

- Normal signal intensity of gray and white matter is noted. No evidence of any focal area of abnormal signal intensity.
- **Intra-cerebral and extra-cerebral CSF spaces appear prominent.**
- Pons, mid-brain and medulla oblongata appear normal.
- No evidence of intra-cerebral, sub-dural, extra-dural bleed.
- Cavernous, sagittal, straight and transverse sinuses appear normal.
- Pituitary gland is normal in size with central infundibulum.
- The vestibulo-cochlear nerve complexes appear normal on both sides.
- Lacrimal glands are normal on both sides.
- **Mucosal thickening is seen in bilateral frontal, ethmoid and right sphenoid sinuses appearing hyperintense on T2WI suggestive of sinusitis.**

CONCLUSION

- **Cerebral atrophy**

Dr. Mudassar Bajwa

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Mall Road, Rawalpindi
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Name: S/O Mr Jahanzaib	ID No. 12881	Age: 21 Yrs	Sex: M
Unit: —	Ward: —	Date: 8.2.2017	

PLAIN MRI CERVICODORSAL SPINE

CLINICAL DATA

- Cerebellar ataxia, improper speech

EXAMINATION TECHNIQUE

- Multi-plane imaging done through cervical spine acquiring T1/T2 weighted sagittal and axial sequences. MR myelography was also performed. Dorsal spine upto DV9 is included in the study.

FINDINGS

- Cervical spine shows normal curvature. The cord appears normal with no evidence of any abnormal expansion or signal intensity.
- **Focal fatty deposit is seen in the vertebral body of DV8.**
- Inter-vertebral discs are normal with no protrusion or herniation. Nucleus pulposus and annulus fibrosis show normal signal intensity.
- Lateral recesses are not narrowed.
- Ligamenta flava and extra-dural fat are within normal limits.
- Neural foramina are normally visualized.
- No evidence of any extra- / intra-dural or paravertebral mass seen.
- Pre-vertebral soft tissues are normal.
- MR myelogram shows no obstruction of CSF column.

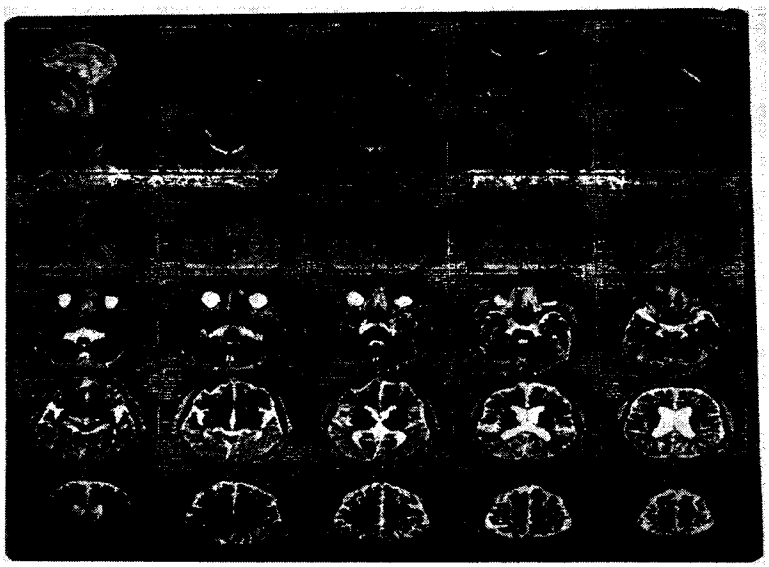
CONCLUSION

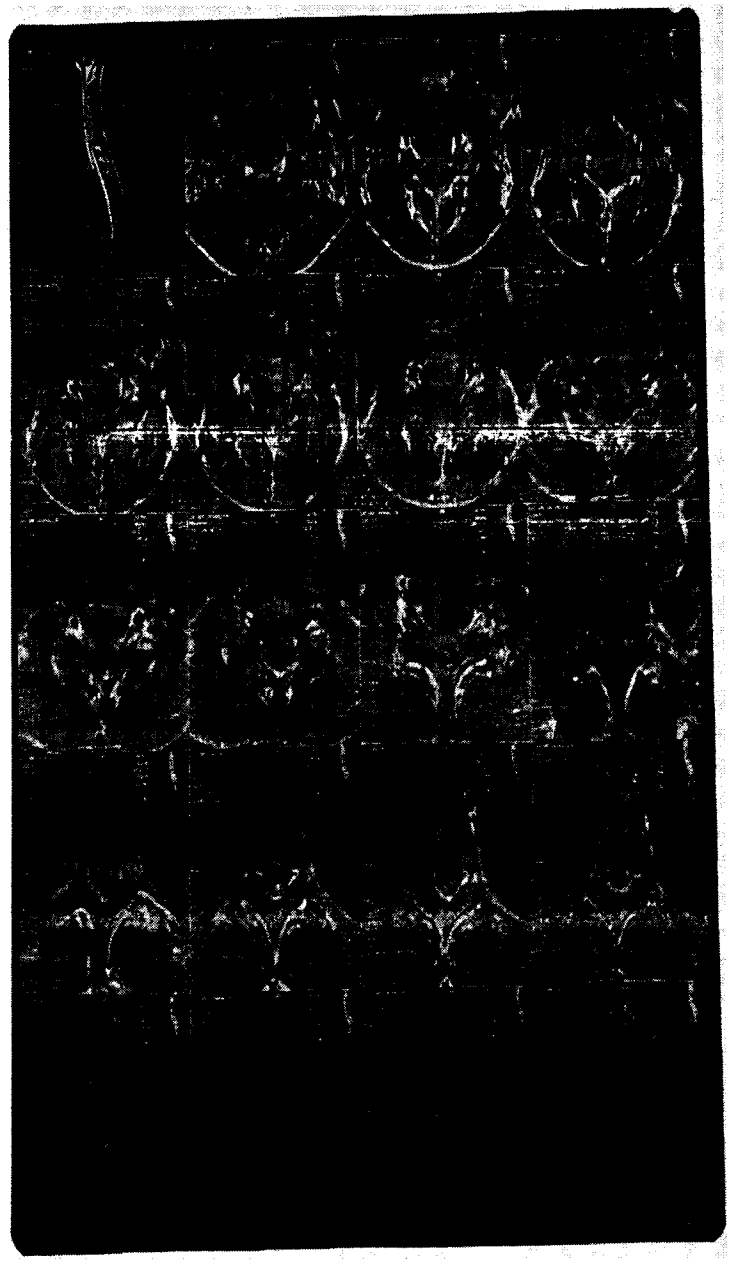
- **Essentially normal study**

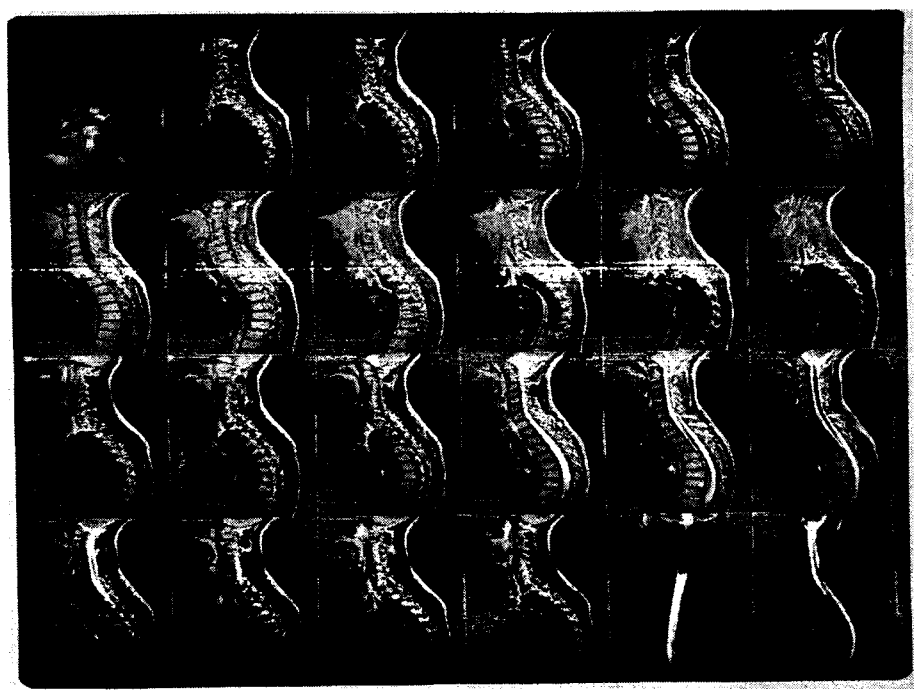
Dr. Mudassar Bajwa

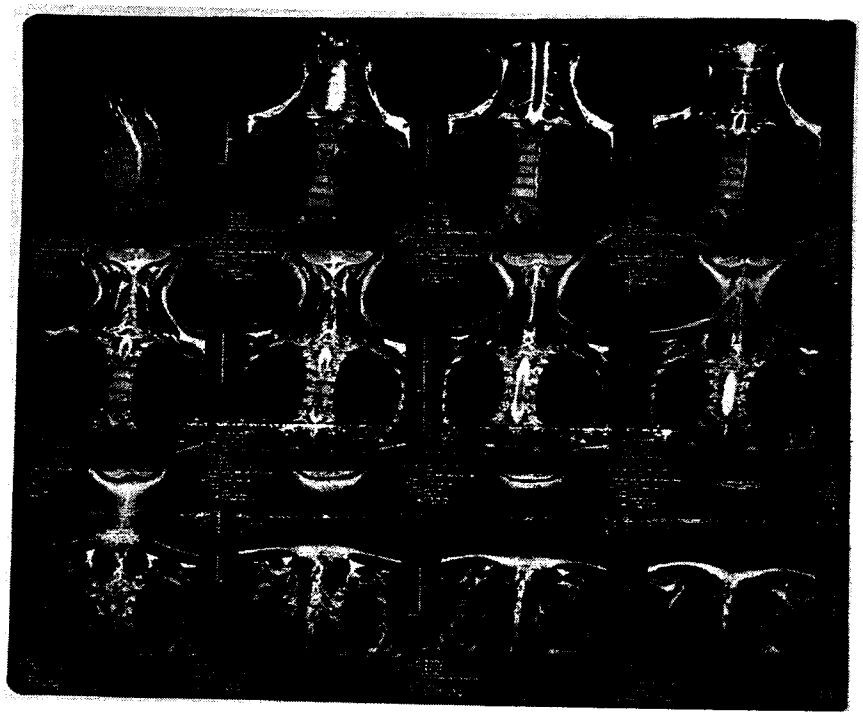
MRI BRAIN, CT SCANAND NEUROIMAGES

FAMILY D.

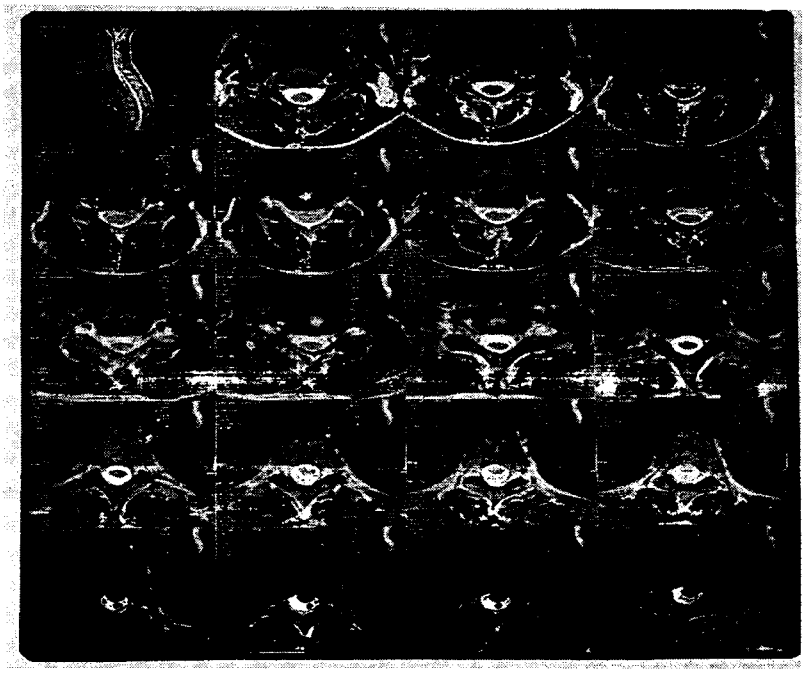


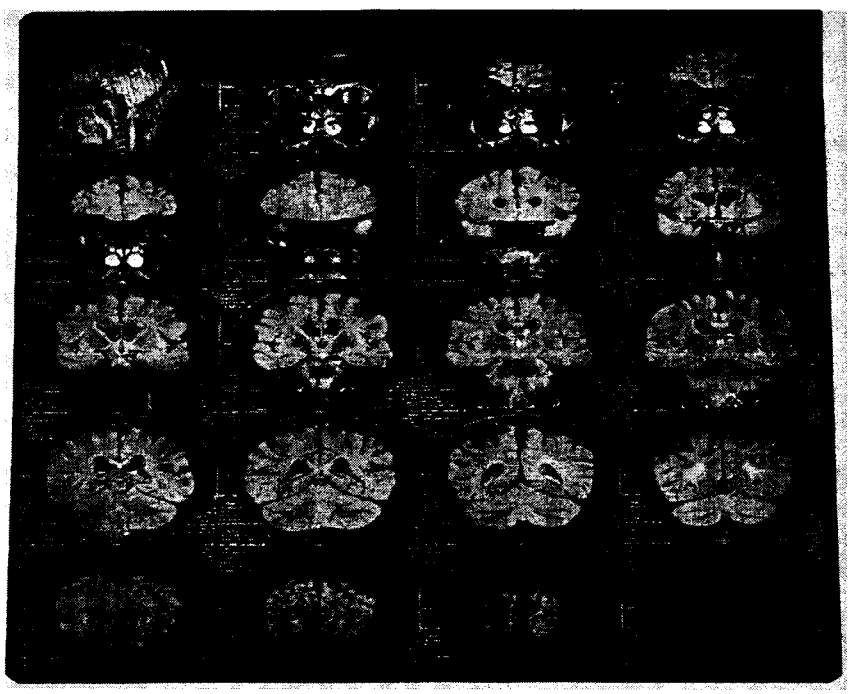


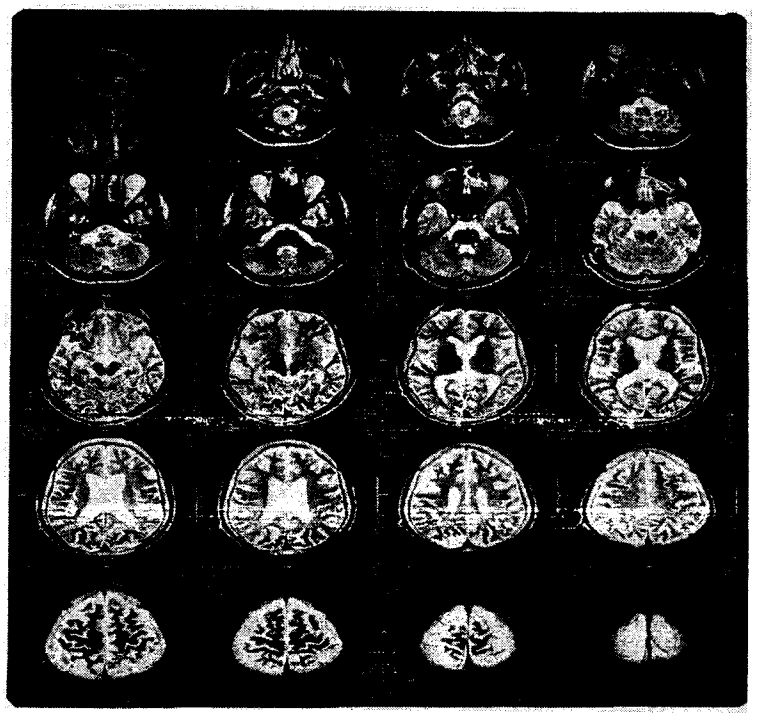




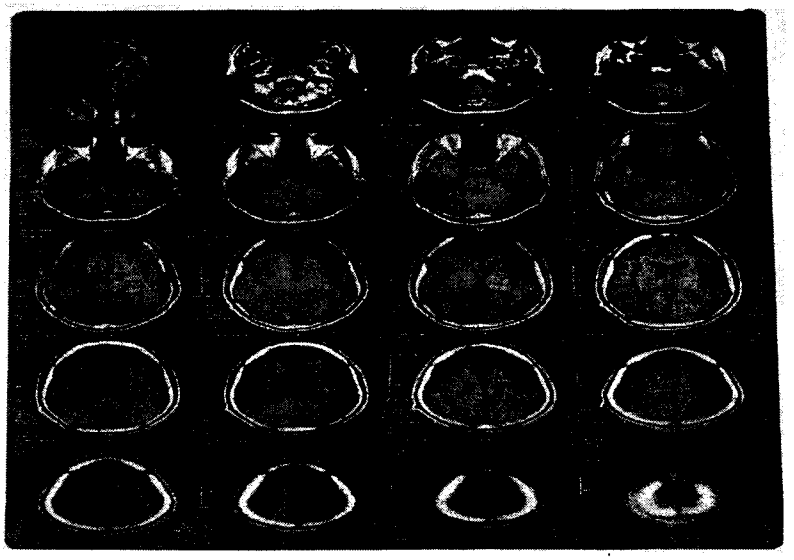
Family E AND F







FAMILY G



FAMILY H

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Regt No.	Rank & Name	Age	Sex	Date
23724	S/O MR JAHANZEB	18Y	M	4-4-14

MRI BRAIN

CLINICAL DATA
Vertigo
x/c Fr

EXAMINATION TECHNIQUE
Multi-planar scanning done through brain acquiring T1, T2 weighted and proton density sequences

FINDINGS
- Normal signal intensity of gray and white matter is noted. No evidence of any focal area of abnormal signal intensity.
- Cerebellum appears normal with no abnormal signal intensity focus.
- No evidence of any SOL brain, intra cerebral bleed or infarction.
- Intra-cerebral and extra-cerebral CSF spaces are within normal limits.
- Pons, mid-brain and medulla oblongata appear normal.
- No evidence of intra-cerebral, sub-dural / extra-dural bleed.
- Cavernous, sagittal, straight and transverse sinuses appear normal.
- Pituitary gland is normal in size with central infundibulum.
- The vestibulo-cochlear nerve complexes appear normal on both sides.
- Optic nerves, optic tracts and optic chiasma appear normal.
- **Hyperintense signal is noted in both maxillary sinus, ethmoidal , sphenoid and right frontal sinuses suggestive of sinusitis.**
- **Hypertrophy of left inferior nasal turbinate is noted.**

CONCLUSION
- **Normal MR study brain** ✓
  No evidence of any SOL brain, intra cerebral bleed or infarction.

MALIK MUHAMMAD KHALID
CLASSIFIED RADIOLOGIST

DEPARTMENT OF RADIOLOGY Date 10-07-2017

Army Medical Complex

MILITARY HOSPITAL

Mall Road

Rawalpindi

Phones: 5613439?, 5613186?

Regt. no.	Svc No.	Rank	Name
54861			Sheraz Khan

Age	Sex	Unit	Wd/ OPD	Date
15 YRS	M		OPD	10-07-2017

MRI LUMBAR SPINE

CLINICAL DATA

- Backache

EXAMINATION TECHNIQUE

- Multi-planar imaging done through lumbar spine acquiring T1,T2 weighted sagittal and axial sequences

FINDINGS

- Spinal cord appears normal with no evidence of any area of abnormal signal intensity in it.
- No evidence of any extra-dural / intra-dural mass.
- Normal appearance of vertebral bodies and posterior elements of spine in the scanned area.
- Inter-vertebral discs appear normal.
- No disc protrusion or herniation is seen in the spinal canal.
- Lateral recesses appear normal.
- Ligamenta flava and the extra-dural fat appear normal in thickness.
- No para-vertebral mass seen.
- Pre-vertebral soft tissues appear normal.
- No pre-vertebral enlarged lymph nodes are seen.

CONCLUSION

- NORMAL STUDY.

BRIG ABDUL GHAFFAR

Family No.	
UCL Lab. No	
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Mobile: II	
Landline:	
Address:	Sahiwal
Booked By	T

Sufyan (05 years)

Regression of Mile Stones: Regression of milestones and ataxia

Speech / Hearing / Vision: NAD

Neurological Assessment: Tone: Increased, Power 4-5/5, Reflexes: Increased, Planters: Up going.

Systemic Review: N.A

Metabolic work up: N.A

MRI Brain: Cerebral atrophy with foci of demyelination in periventricular white matter.

EEG: Multifocal epileptiform activity

Ophthalmologist Notes:

EMG/NCS AND Diagnosis: Cerebral hypoplasia and gait difficulties

Family I AND J