Genetic Analysis of Epilepsy in Consanguineous Families

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CERTIFICATE

This thesis, submitted by **Mr. Hamza Hameed** to the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the Degree of Master of Philosophy in Biochemistry/Molecular Biology.

Examination Committee:

1. **External Examiner:** Signature: _____

2. **Supervisor:**
   Prof. Dr. Muhammad Ansar Signature: 

3. **Chairperson:**
   Prof. Dr. Iram Murtaza Signature: 

Dated:

Declaration

I hereby declare that the work presented in the following thesis is my own effort and the thesis is composed by me. No part of this thesis has been previously presented elsewhere for any other degree or certificate.

Hamza Hameed

Dedicated to

MY

Father

Who believed in me and has

Always been my support and

MY

MOTHER

Whose prayers and

Love is endless

Acknowledgments

I literally have no words to express my gratitude to the One who has the hold on my life, ALLAH ALMIGHTY, the Most Beneficent, the Most Merciful. All the praises and glories be to Almighty Allah the Lord of the universe for His infinite mercy and blessings upon us. I offer my salutations to Holy Prophet Muhammad (PBUH), who has always been my greatest source of inspiration throughout my life. I am honored to be a part of his ummah. I am deeply thankful to him for the moral lessons he taught us.

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Hamza Hameed

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List of Abbreviations	Description
WHO	World Health Organization
CNS	Central Nervous System
ILAE	International League Against Epilepsy
ID	Intellectual Disability
EEG	Electroencephalography
CMV	Cytomegalovirus
HIV	Human Immunodeficiency Virus
MRI	Magnetic resonance imaging
CT	Computed tomography
BOLD	Blood oxygen level dependence
fMRI	Functional magnetic resonance imaging
PET	Positron emission tomography
SPECT	Single-photon emission-computed tomography
GLUT1	Glucose transporter 1
CSF	Cerebrospinal Fluid
EDTA	Ethylenediamine Tetra-acetic Acid
SDS	Sodium Dodecyl Sulphate
TE	Tris-EDTA
DMSO	Dimethyl sulfoxide

MgCl2	Magnesium Chloride
NaCl	Sodium Chloride
AEDs	Antiepileptic drugs
DS	Dravet Syndrome
CNV	Copy number variation
CGH	Comparative genomic hybridization
CADD	Combined Annotation Dependent Depletion
ESE	Exon splicing enhancer
ESS	Exon splicing silencer
UTR	Untranslated Region
C-section	Cesarean section
TGP	TranGene Promoters
ACMG	American college of Medical Genetics and Genomics
SCN1A	Sodium voltage-gated channel alpha subunit 1

Abstract

Epilepsy is a neurological disorder, which is also referred as seizure disorder. It is mainly characterized by recurrent seizures and is classified into different types depending on the clinical features and the type of seizure presented by the respective patient. Approximately 168 genes have been identified in patients with isolated epilepsy. Out of these, 90 genes (53%) are involved in causing autosomal dominant epilepsy and 62 genes (32%) are known for autosomal recessive epilepsy. However, mutations in some genes (SCN1A, KCNQ2, STXBP1, KCNQ3, DEPDC5, SCN2A, PRRT2, DEPDC5) have been identified in both autosomal dominant and recessive epilepsy. The clinical features of patients from five families included the presentation of tonic clonic seizures. All five families showed autosomal recessive inheritance of epilepsy, and the presented clinical features resemble Dravet syndrome, which is mainly caused by mutation in SCN1A gene. Blood was drawn from all family members but only one affected member from each family was selected for Sanger sequencing of SCN1A gene. Five exons of SCN1A gene were selected including exon 5, 15, 21, 25, and 26. The analysis of Sanger sequencing data detected a homozygous polymorphism g.19189-19190insG in family B which lies in the intron 5, one variant of unknown significance i.e., g.82579A>G in family B and D which lies in 3ʾuntranslated region (3ʾUTR) of exon 26, and a homozygous polymorphism g.64092_64093isnA in intron 21 of family D. The g.82579A>G is identified in two families (B, D) in the homozygous state and is predicted as disease causing by mutation taster. However, additional studies are required to explore the functional effect of this variant. No variant was identified in family A, C and E. In conclusion, a potential variant was identified in two families, but further research will be required to find the disease-causing variants in the remaining three families.

Keywords: Epilepsy, seizures, Dravet syndrome, SCN1A

1. Introduction

Epilepsy, referred to as seizure disorder, is a neurological disorder which is mainly characterized by recurrent and unprovoked seizures. Epilepsy characterized by predisposition to experience recurrent seizures (Falco et al., 2020). A "seizure" is an abrupt alteration in neurologic function results from an excessive discharge of positive signals (continuous firing of the neurons) of the neurons in the brain (Giourou et al., 2015). According to epidemiological studies approximately 400 to 500 lac people in the world suffer from epilepsy (McTague et al., 2016). Moreover, epilepsy is more commonly found in elderly and infancy. The term "Epileptic seizure" is employed to differentiate typical seizure triggered by abnormal neuronal activity from non-epileptic events, such as a psychogenic seizure (Shorvon et al., 2011).

1.1. Symptoms of Epilepsy

Seizure symptoms can vary based on specific type of seizure. Since epilepsy arises from changes in electrical activity of the brain, can affect many brain processes. Seizure symptoms include, muscles stiffness, continuous irregular movement of the arms and legs, temporary confusion, psychological symptoms such as fear and anxiety. Feeling something in stomach, loss of consciousness, loss of balance, and dizziness (Stafstrom et al., 2015). To define the type of epilepsy, we first need to understand seizure types.

1.2. Types of Seizures

There are multiple types of seizures based on the duration of seizures, but these are mainly classified as either Focal seizures, (experienced by 70% of people with epilepsy) or generalized seizures (Scheffer et al., 2017).

1.2.1. Focal Seizures

Focal seizures also recognized as partial seizures, are primarily identified by irregular electrical activity in specific area of the brain referred to as the 'Focus'.

1.2.1.1. Clinical manifestation of Focal Seizures

Clinical manifestations are largely determined by the cortical area involved in particular type of epilepsy. The focal seizure originates from:

i) The occipital lobe of brain, potentially giving rise to visual phenomenon.
ii) The precentral gyrus, with tonic motor or rhythmic activity
iii) The postcentral gyrus giving rise to sensory symptoms.

When consciousness is compromised during focal seizure, the individual does not exhibit a typical response to verbal and tactile stimuli. These seizures are commonly categorized as complex partial seizures, originating from temporal lobe, and are often associated with altered awareness. In certain focal seizures, patient maintain awareness and can articulate motor, sensory, or autonomic symptoms, referred to as aura. An aura precedes a focal dyscognitive or generalized seizure by several seconds or minutes and is frequently reported by individuals with temporal lobe epilepsy (Stafstrom et al., 2015).

1.2.1.2. Signs and Symptoms of Focal Seizures

Most of the people with focal seizures mainly have signs in the moments just before seizure starts. These signs are called aura. Some warning signs include feeling something in the stomach and sometimes emotions as fear. These auras can also be a taste or a smell. It can be visual, such as flashing light, a color, or a shape. Most people experience dizziness and loss of balance. Sometimes people see things that aren't there at that time which is also known as hallucinations (Engel et al., 2010).

Focal seizures are further sub categorized in;

1.2.1.3. Focal seizures without loss of consciousness

These are alternatively termed as simple partial seizure since they don't cause a loss of awareness, also referred to as consciousness. These seizures can change a person’s emotion or alter the way things look, smell, feel, taste or sound. This seizure results in involuntary shaking of legs and arms. Focal seizures lead to sensory symptoms such as dizziness, flashing lights and tingling (Johnson et al., 2019).

1.2.1.4. Focal seizures with impaired awareness

These are also referred to as complex partial seizures and they involve state of unconsciousness. These types of seizures may experience dream like state. While undergoing a focal seizure with unconsciousness, people may not be able to respond to the environment in typical way. Some people may also perform irregular continuous movements, such as chewing, rubbing their hands, walking around, swallowing (Liu et al., 2020).

Focal seizures can arise from any cerebral lobe (Fig 1.1). Types of focal seizures include:

1.2.1.5. Temporal lobe seizures

Temporal lobe seizures originate in the temporal lobes. Temporal lobe plays important role in short-term memory and process emotions. Individual undergoing these seizures frequently encounter sudden fear or joy. They have rising sensation in their stomach. As seizures start individuals become unawareness of their surroundings. They may gaze into space, have movements of their fingers, start swallow, or chew repeatedly (Abarrategui et al., 2021).

1.2.1.6. Frontal lobe seizures

Frontal lobe seizures originate in front part of the brain. This region of the brain governs movement. Seizures originating in the frontal lobe lead individual to turn their eyes and head in one direction. They remain unresponsive and may laugh or scream. They can also extend their one arm and flex the other, as well as engage in repetitive movements like rocking and pedaling bicycle.

1.2.1.7. Occipital lobe seizures

Seizures originate in occipital lobe, a region associated with vision. Individual experiencing this seizure undergo hallucination, or encounter full or partial vision loss. These seizures may also induce episodes of eye blinking (Traianou et al., 2019).

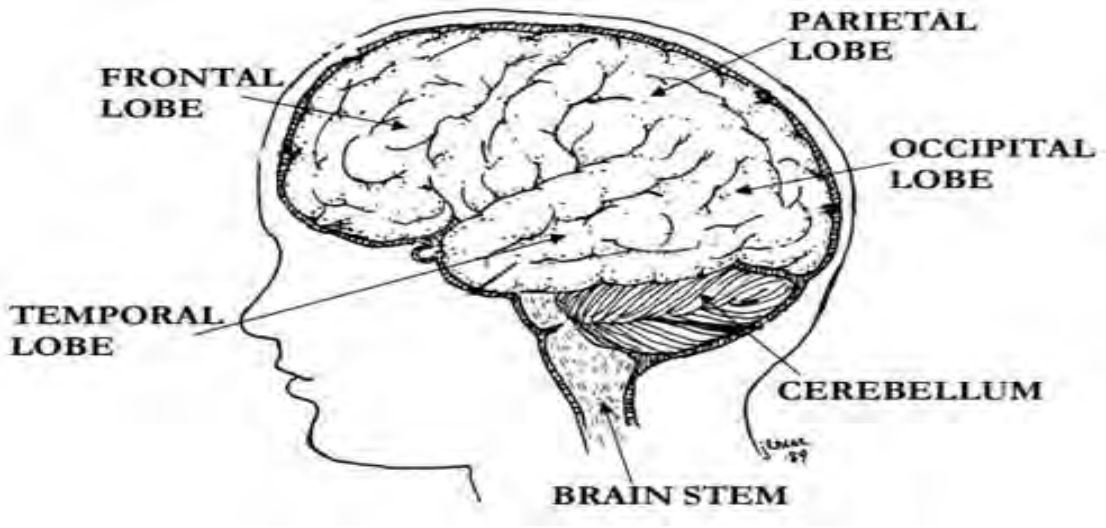

Figure 1.1 Focal seizure effecting parts of Brain. Adopted from https://www.nationwidechildrens.org/conditions/seizures-focal-partial.

1.2.2. Generalized seizures

Seizures that appear to involve all areas of the brain are called generalized seizures. These seizures cover both section of central nervous system which are sub-categorized into absence, atonic, tonic clonic and myoclonic seizures.

1.2.2.1. Absence Seizures

These are also called as petit mal seizures. These seizures most commonly occur more than one time a day for about 2 -15 seconds. In most of patients it was observed to occur around 100 times per day. They cause non-descript stare by person having seizures (Crunelli et al., 2020).

In case of absence seizure, a person experience daydreaming, have difficulty during picking something or jerky movement in whole body. These seizures generally occur in children within range of 4 to 12-year-old and sometimes found in person above the age of 20 years (Hirsch et al., 2022).

1.2.2.2. Generalized Tonic-Clonic Seizure

These are also classified as convulsive seizures or grand seizures. A person who is having this type of seizures might have observed with contractile and spasmic condition

in their arms and legs and body stiffening called tonic stage. While in clonic stage, person have jerky movements in their limbs and head. These stages might change themselves based on tonic-clonic phase. When the seizure occur, person can be seen as biting their own tongues, ceasing in their breathing, uncontrolled movements (Asadi et al., 2020).

1.2.2.3. Myoclonic seizures

Myoclonic seizures can be defined by jerky movement of the body like irregular movements or twitching of arms or legs. These movements are mostly observed during nighttime rather than the daytime when a person is asleep. Person having these seizures might feel them as the graceless behavior and most commonly it occurs in upper body parts (Chandarana et al., 2021).

1.2.2.4. Atonic seizures

These seizures are also named as drop attacks or astatic seizures. Persons having seizures might experience swing behavior, during this a person might drop suddenly or the person might collapse totally. Because of unique body movements in this, it is considered very dangerous. A person might have complete loss of control on muscles (Oguni et al., 2022).

1.2.3. Simple Partial Seizure

In case of simple partial seizure, a person remains conscious and completely alert while epilepsy is going on. But he/she will not be able to move and speak until the process completed. Depending on the part of CNS involved, person may have uncontrolled eye movement, irregular hands or feet movement and fast-continuous blinking of their eyes. Most commonly it involves body movement in a defined manner. In this situation person will see and hear things which are not occurring at that time (Sriraam et al., 2019).

1.2.4. Complex Partial Seizures

Complex partial seizures affect more than single half of CNS. It shows some pathological effects on one part of the temporal lobes. This type of seizure is also known

as temporal lobe epilepsy (TLE), and patients don't have enough power to do their work. These people have monotonous and chewing movements. They also have unsystematic movements (Tiglani et al., 2020).

1.3. Classification of Epilepsy

The International league against epilepsy (ILAE) classified epilepsy, on the basis of etiology at each step of diagnostic process (Scheffer et al., 2017). To classify epilepsy, seizure types are considered along with information about clinical presentation, imaging results, laboratory tests, comorbidities, genetic factors and prognoses. ILAE classified epilepsy, into three levels (Fig 1.2) which are explained below:

Level 1: Firstly, the type of seizure is defined. There are three types of seizures based on their origin. i) Focal Seizures which begin within specific brain regions and are limited to only one hemisphere. ii) Generalized Seizures which originate at some point within and then bilaterally distribute in brain regions. iii) Seizures of unknown onset, if the information regarding the type of seizure (focal or generalized) is insufficient.

Level 2: Level 2 includes diagnosis of the kind of epilepsy. There are four main classes: Focal epilepsy, generalized epilepsy, unknown epilepsy and combined focal and generalized epilepsy. It includes combination of generalized and focal epilepsy which reflect the shared mechanisms (Fisher et al., 2017).

Level 3: In Level 3 diagnosis of specific epilepsy syndrome is carried out.

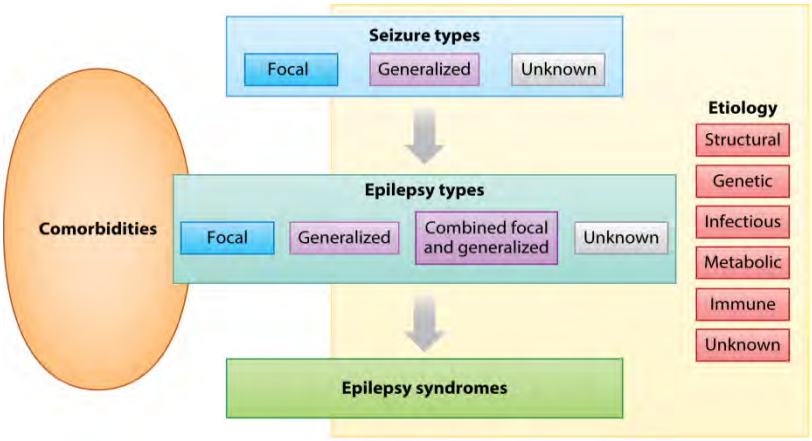

Figure 1.2. Framework for classification of epilepsies. Adopted from Perruca et al., 2020.

1.4. Etiology

Epilepsy has diverse underlying causes. Any factor that can cause disruption of normal electrical pattern in brain can result in epileptic seizures. Onset of epilepsy can results from either genetic or environmental factors. Tumors, head injuries, stroke, and damage to brain before birth because of maternal infection or cerebral hypoxia can also lead to epileptic seizures. Epilepsy is also frequently observed in individuals with developmental disorders including, Intellectual disability (ID), microcephaly and autism.

The ILAE has established six etiologic categories, with specific emphasis on those causes that carry consequences for management of epilepsy. Categorization of these etiologies is given below:

1.4.1. Structural Etiology

If neuroimaging result of an individual is not normal and concordant with the symptoms and EEG, then it can be concluded the cause of seizure is this abnormality. Nevertheless, if the neuroimaging result is different from the individual's symptoms and EEG, then this abnormality is not related to epilepsy. (Berg et al., 2007).

1.4.2. Genetic Etiology

Genetic forms of epilepsy result from mutations in the genes encoding receptors or ion channels of the neurons in the brain. If an individual possesses a strong family history and characteristic features such as seizure characteristics and EEG then genetic etiology of epilepsy is confirmed without the need of information regarding molecular genetics. However, certain disease-causing variants can arise spontaneously (de novo) and are not inherited, so even in the absence of family history, an individual could still possess a genetic predisposition to epilepsy (Scheffer et al., 2017).

1.4.3. Infectious Etiology

Brain infections (bacterial or viral) such as neurocysticercosis, CMV, HIV, cerebral toxoplasmosis can cause epilepsy. Seizures because of a previously cured infection such as meningitis would also fall in the category of infectious etiology (Vezzani et al., 2016).

1.4.4. Metabolic Epilepsy

This refers to individuals with epilepsy in which the primary cause is metabolic derangement. Individuals experiencing acute-symptomatic seizures due to a transient metabolic disturbance are not included in this category. Although, majority of epilepsies have a genetic origin, although some are acquired. Examples of metabolic epilepsies are pyridoxine-dependent seizures and cerebral folate deficiency (Scheffer et al., 2017).

1.4.5. Immune Etiology

Epilepsy is also frequently observed in individuals with autoimmune diseases (Vezzani et al., 2016). Limbic encephalitis has been recognized as a cause epilepsy in many individuals with epilepsy of unknown origin (Lancaster & Dalmau, 2012).

1.4.6. Unknown Category

This category encompasses individual with an unidentified cause of their epilepsy.

1.5. Diagnostic Evaluation

1.5.1. History and Examination

History of the individual and neurological examination are important for diagnosis of epilepsy. While taking details about type of seizure, certain neurological examinations are always required for focal signs to identify underlying pathology.

1.5.2. Electroencephalography (EEG)

An EEG can record brain electrical activity to identify irregularities, such as bilateral spike waves indicative of generalized epilepsy or focal spikes suggestive of focal epilepsy, as epileptiform abnormalities vary across different states of consciousness, a routine EEG is always required which deals to include wakefulness, sleep and drowsiness. To increase the overall yield of epileptic activity, two such procedures are carried out such as Hyperventilation and photic stimulation (Bulus et al., 2022).

Hyperventilation for 3 minutes gives high yield towards an absence seizure that relate alkalosis to induce epilepsy (Schuchmann et al., 2006). In individuals prone to generalized epilepsy, photonic stimulation has potential to trigger a generalized seizure (Verrotti et al., 2012). Continuous EEG monitoring for extended period of time ranging from hours to days can help to distinguish an epileptic seizure from a nonepileptic events (Husain et al., 2021).

If seizures originate from frontal or temporal lobe, patient's EEG can be repeatedly normal. Intracranial EEG is preferred in such cases which is necessary to define a seizure focus (Jobst et al., 2020).

1.5.3. Neuroimaging

Computed tomography (CT) and Magnetic resonance imaging (MRI) scans are the two most compulsory test for clinical examination. These neuroimaging techniques exhibit high sensitivity in detecting structural lesions with the central nervous system (CNS).

MRI is commonly employed to reveal abnormality in individuals experiencing focal seizures and to demonstrate abnormal focal discharge patterns identified in EEG

(Gelisse et al., 2021). MRI is preferred over CT because of more sensitivity, particularly effective in identifying cortical malformation or hippocampal sclerosis (Wang et al., 2020). Asymmetries of temporal lobe can be detected through Computer-assisted volume analysis that may not be evident through visual examination of the scan. Functional MRI (fMRI) measures the blood oxygen level dependence (BOLD) which images epileptiform activity and used to localize memory and language (Bisdas et al., 2019).

Magnetic resonance (MR) spectroscopy is used to quantify concentration of certain neurochemicals in various regions of the brain and help identify location of a particular seizure. Positron emission tomography (PET) scan depict the regional utilization of glucose in the brain, revealing asymmetries that indicate various areas of abnormality during interictal (between seizures) or ictal (during seizure) abnormalities (Zhang et al., 2023). Single photon emission computed tomography (SPECT) can compare the local blood flow. Magneto encephalography (MEG) is utilized to capture the dynamic electromagnetic fields of brain which cannot be taken up by EEG and are helpful to locate epileptic dipoles, especially those tangential to scalp (Caruso et al., 2013).

1.5.4. Metabolic Evaluation

It provide detail of syndrome and the type of seizure which helps in metabolic investigation (Pearl 2009). Individuals with Lennox Gastaut syndrome, a severe type of epilepsy it is most probable that they have a degenerative or metabolic disorder compared to individual having simple partial seizure (Na et al., 2020).

In most metabolic disorders, seizures occur along with other abnormalities, like coma, vomiting and developmental delay. Individual having neonatal seizure, require metabolic evaluation to screen out mitochondrial diseases, including urine organic acids, blood lactate and serum amino acids. Glucose transporter defects (GLUT1 deficiency syndrome) can be examined by measuring very rare neurotransmitter defects (Pearl et al., 2007) and cerebrospinal fluid (CSF) (Pearson et al., 2013).

1.5.5. Genetic Testing

The epilepsies genetics are increasingly being unrevealed and clinical testing can play vital role in diagnosis (Michelucci et al., 2012). Genetic testing is accessible for single gene disorder and complex genetic disorders (Vezzani et al., 2015). If a particular syndrome or epilepsy is suspected, one can request a collection of specific genes e.g., *SCN1A* for Dravet syndrome [DS].

The chromosomal regions having specific copy number variation (CNV), can be identified by comparative genomic hybridization (CGH) microarray. In negative cases genetic diagnosis can involve whole exome sequencing. (Olson et al., 2014).

1.6. Treatment of Epilepsy in Pakistan

In Pakistan, different types of therapies are available, including medicines and surgical procedures.

1.6.1. Medication

Antiepileptic drugs (AEDs) are the first line of treatment against epilepsy. Mostly, Carbamazepine, phenytoin, lamotrigine drugs are used to control seizures (Gierbolini et al., 2016)

1.6.2. Low-Level Laser Therapy

This therapy is available in various centers of Pakistan. It has been proven to be effective in multiple epilepsy patients (Siddiqui et al., 2015).

1.6.3. Vagus Nerve Stimulators

This is a surgical procedure that is implanted in the left vagus nerve to calm down the frequency and intensity of seizures (Gonzalez et al., 2019).

1.6.4. Advanced Neurosurgical procedures

A very less invasive neurosurgery called temporal lobectomy, is carried out to remove the temporal lobe. Temporal lobes produce abnormal electrical activity that leads to

seizures (https://www.shifa.com.pk/epilepsy-treatment-through-advanced neurosurgical-procedure/).

1.7. Genetics of Epilepsy

The inherited types of epilepsy can be caused by mutations in ion channel genes, as well as enzyme/enzyme-modulator genes (Wang et al., 2017). The inherited types of epilepsy can be categorized as autosomal dominant or autosomal recessive based on pattern of inheritance. Autosomal dominant epilepsies are mostly monogenic, where a single mutant allele of gene is responsible for the condition, and these are typically observed in extensive familial groups (Kaneko et al., 2003). Familial focal epilepsy with variable foci (FFEVF), Benign familial neonatal seizures (BFNS), Generalized epilepsy with febrile seizures plus (GEFS+) are some common examples of autosomal dominant epilepsy. In the case of autosomal recessive epilepsy an individual must inherit two copies of mutant gene associated with epilepsy, one from each parent. This type of epilepsy often results from combination of recessive genetic factors and Amish infantile epilepsy syndrome (AIES) is the common example. Some inherited types of epilepsy show both autosomal dominant and recessive pattern of inheritance, this include Dravet syndrome (DS), Early infantile epileptic encephalopathy (EIEE), Familial febrile seizures (FFS), and Progressive myoclonic epilepsy (PME).

The data obtained from OMIM database, approximately 1506 genes have been identified as associated with epilepsy, categorizing them into three groups based on their connection to epilepsy and related syndromes. Out of these 168 genes were linked with pure epilepsy, 364 genes were connected to neurodevelopmental disorders accompanied by epilepsy, while 974 genes were associated with physical abnormalities preceding epilepsy. A list of linked genes with different forms of epilepsy without additional abnormalities is provided in table 1.1.

Total number of genes reported for epilepsy without any neurodevelopmental or structural disorder are 168. Out of these 90 genes (53.6%) show autosomal dominant inheritance and 62 genes (36.9%) show autosomal recessive inheritance (Zhang et al.,2023

Some examples of genes involved in autosomal dominant cases of different types of inherited epilepsy include CHD2, GRIN2A, CPA6 while genes like PNPO, PLPBP, and ALDH7A1 are reported in autosomal recessive cases (Table 1.1). However, mutations in a few genes are reported for both autosomal dominant and recessive types of epilepsy including SCN1A (Lal et al., 2016; Marco et al., 2021), SCN2A (Corbett et al., 2016 and AlSaif et al., 2019), KCNQ2 (Perucca et al., 2019; Kuersten et al., 2020), KCNQ3 (Allen et al., 2014; Lauritano et al., 2019), STXBP1 (Khaikin et al., 2016; Balagura et al., 2022), PRRT2 (Zhao et., 2020; Musa et al., 2023), SLC2A1 (Klepper et al., 2009; Algahtani et., 2020) and DEPDC5 (Picard et al., 2014; Myers et al., 2017). SCN1A is the most frequently mutated gene with (70-80%) mutations linked to DS, a type of epileptic syndrome (Scheffer et al., 2019). Almost 16% of epileptic cases are due to variants in PCDH19, SCN1B, SCN2A, and GABRG2, STXBP1, HCN1, and CDH2 genes (Ikram et al., 2022). CDKL5 and ARHGEF9 show X-linked recessive and X-linked dominant pattern, respectively. Additionally, EFCH1 is involved in 10-30% cases (Saleem et al., 2021), while other genes SCN2A, STXBP1, SCN8A are reported.

Table 1.1: Genes reported in inherited types of epilepsy.

Phenotype	AD Genes	AR Genes
Benign familial neonatal seizures (BFNS)	*PRRT2*, *SCN2A*, *SCN8A,KCNQ2, KCNQ3*	*SCN2A, KCNQ2, KCNQ3*
Amish infantile epilepsy syndrome (AIES)		*ST3GAL5*
Pyridoxine-dependent epilepsy		*ALDH7A1*
Paroxysomal kinesigenic dyskinesia and epilepsy	*PRRT2,SCN1A, SCN2A*	*PRRT2*
Early onset epilepsy and intellectual disability	*STXBP1,CDKL5, SLC2A1*	*STXBP1*

Early infantile epileptic encephalopathy (EIEE)	*CACNA1A, GABRA1*, *GABRB3*, *KCNQ2*, *KCNT1*, *SCN2A*, *SCN8A*	*AARS*, *ARV1*, *DOCK7*, *FRRS1L*, *GUF1*, *ITPA*, *NECAP1*,*PLCB1*, *SLC12A5*, *SLC13A5*, *SLC25A12*, *SLC25A22*, *ST3GAL3*, *SZT2*, *TBC1D24*, *WWOX*
Dravet syndrome (DS)	*SCN1A, SCN9A*	SCN1A
Focal Epilepsy	*DEPDC5*	*DEPDC5*
Familial febrile seizures (FFS)	*GABRG2*, *GPR98*, *SCN1A*, *SCN9A*	*CPA6*
Generalized epilepsy with febrile seizures plus (GEFS +)	*GABRD*, *GABRG2*, *SCN1A*, *SCN1B*, *SCN9A*, *STX1B*	
Generalized epilepsy and paroxysmal dyskinesia (GEPD)	*KCNMA1*	
Myoclonic-atonic epilepsy (MAE)	*SLC6A1*	
Childhood-onset epileptic encephalopathy (COEE)	*CHD2*	
Focal epilepsy and speech disorder (FESD)	*GRIN2A*	
Childhood absence epilepsy (CAE)	*GABRG2*	
Juvenile absence epilepsy (JAE)	*CLCN2*, *EFHC1*	
Juvenile myoclonic epilepsy (JME)	*CACNB4*, *CLCN2*, *EFHC1*, *GABRD*	
Idiopathic generalized epilepsy (IGE)	*CACNB4*, *CLCN2*, *GABRD*, *SLC12A5*, *SLC2A1*	
Familial adult myoclonic epilepsy (FAME)	*ADRA2B*	*CNTN2 CPA6*
Familial temporal lobe epilepsy (FTLE)	*CPA6*, *GAL*, *LGI1*	

Progressive myoclonic epilepsy (PME)	*KCNC1*	*CERS1*, *CSTB*, *EPM2A*, *GOSR2*, *KCTD7*, *LMNB2*, *NHLRC1*, *PRDM8*, *PRICKLE1*, *SCARB2, CNTN2 CPA6*
Nocturnal frontal lobe epilepsy (NFLE)	*CHRNA2*, *CHRNA 4*, *KCNT1*	
Familial focal epilepsy with variable foci (FFEVF)	*DEPDC5,NPRL2, NPRL3*	

1.8. Dravet Syndrome

Dravet syndrome (DS) typically manifests between age 1 and 18 months of age, characterized by prolonged seizures often presenting as generalized tonic-clonic seizures. Myoclonic seizures mostly occur after two years of age (Li et al., 2021). These seizures are primarily induced by light stimuli, hyperthermia (such as, fever after vaccination and a hot bath) or sodium channel-blocking medication. Seizures become less in severity after puberty, rarely they resolve completely (Aiba et al., 2023).

Initial EEGs are mostly normal however, with the passage of time, epileptiform activity begins to emerge. EEG patterns exhibit generalized wave discharge and reveal multiple spikes. Brain MRI is usually unremarkable, occasionally displaying mild generalized atrophy (Guerin et al., 2019). Individuals with Dravet syndrome often have some unusual myoclonic seizures and show cognitive dysfunction, ataxia, and psychomotor regression (Rilstone et al., 2012). These cognitive impairments range from moderate to severe (Aljaafari et al., 2017).

Individuals with Dravet syndrome frequently develop a crouched gait. Affected individuals seen to be more mobile. The alteration gait is more commonly observed in in elder children. According to a study showed these changes were absent before 5 years of age, but present in five of ten children age between 6-12 years and in 8 of 9 children age between 13 years or older (Rodda et al., 2012). In one cohort study, five out of ten individuals diagnosed with Dravet syndrome exhibited a crouched gait (Rilstone et al., 2012). Studies have shown almost 90% of individuals with Dravet syndrome possess mutations in SCN1A gene (Claes et al., 2001; Malini et al., 2011). Majority of these

mutations result in formation of truncated protein or are missense or splice site mutations. Large deletions have also been observed in a few patients (Malini et al., 2011). Types of mutations with percentages in SCN1A gene causing Dravet syndrome are depicted in figure 1.3. The remaining 10% cases have been linked to pathogenic mutations in SCN1B, HCN1, GABRG2, KCNA2, GABRA1, and PCDH19 (Steel et al., 2017).

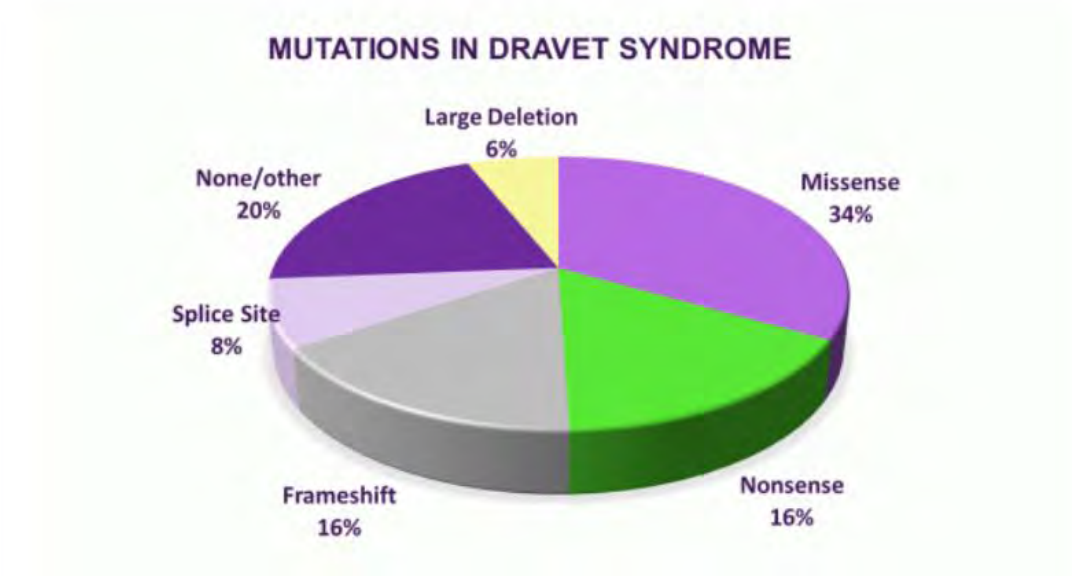

Figure 1.3. Mutations in SCN1A gene causing Dravet syndrome.

1.9. SCN1A

The SCNA1 gene is responsible for encoding the alpha subunit of the voltage-gated sodium channel Nav1. The mutations in this gene are involved in various phenotypes including developmental and epileptic encephalopathies, Dravet syndrome, Febrile seizures, familial, 3A, Generalized epilepsy with febrile seizures plus, and Migraine (Miller et al., 2022).

The sodium channel protein comprised of extensively processed alpha subunit with a molecular weight 260-kDa. The alpha subunit of the sodium channel has four analogous domains named D1, D2, D3, and D4. Each of them further has six transmembrane domains from S1 to S6 (Fig 1.4). A P-loop resembling a hairpin, situated between S5

and S6 constitute a portion of channel pore. Additionally, the intracellular loop linking domains III and IV is involved (Catterall, 2000). Accessory Beta subunits, such as b1, b2, b3, or b4 subunits, are linked to sodium channels in the adult nervous system (CNS) (Dudem et al., 2021).

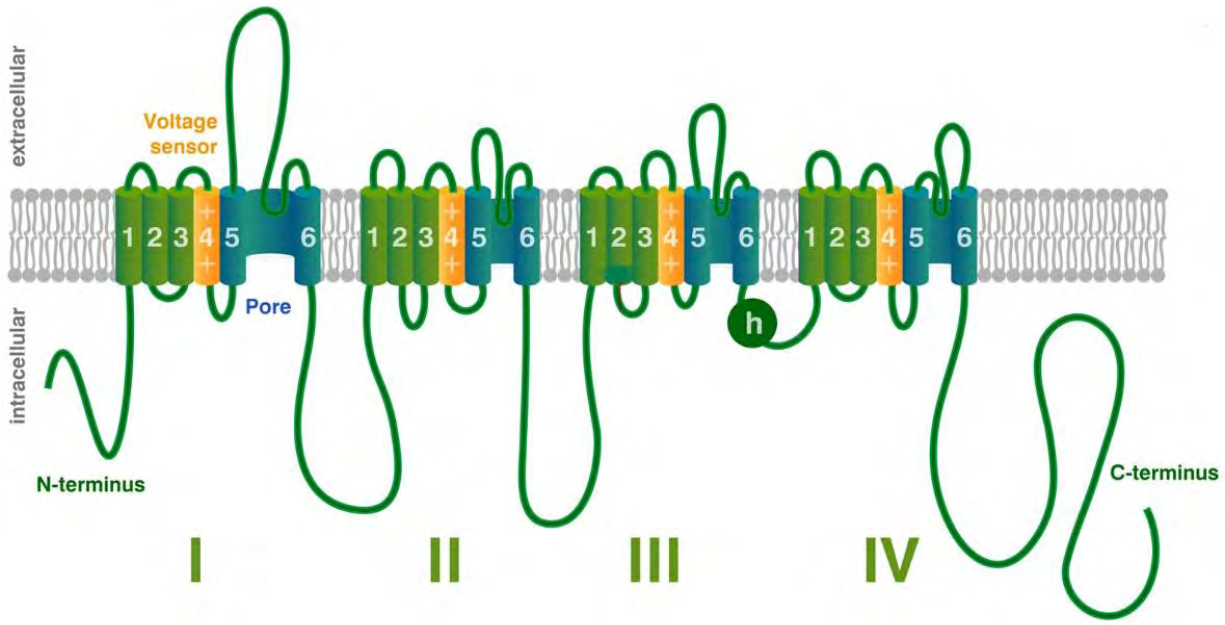

Figure 1.4 Schematic topology of SCNA1, the voltage-gated sodium channels exhibit blue-colored pore-forming region in each domain, with the positively charged S4 voltage-sensing segments are shown in yellow. The above image is adapted and modified from Peter et al., (2016).

The SCNA1 gene is responsible for encoding the alpha subunit of sodium channel NaV1.1, a crucial component in transmitting signals between neurons (Al-ward et al., 2021). These channels are responsible for controlling the flow of sodium (Na) into the neurons. In the D4 loop, a highly conserved P-loop region is present which plays a vital role in the inactivation of the channel (Thompson et al., 2023). Mutations in these regions can result in aberrant channel activity and an increased influx of sodium channels.

Typically, interneurons play a role in inhibiting the generation of electrical activity in brain cells (Fig 1.5). However, when SCN1A variant is present, interneurons lose their ability to suppress electrical activity, ultimately continuous firing of neuron, which causes electrical imbalance in brain cells leading to seizures (Bryson et al., 2021).

Functional studies on mouse models have also demonstrated reduced excitability in inhibitory interneurons because of deletion of Scn1a gene's last exon (Meisler et al., 2021). However, the deletion had no effect on excitability of excitatory pyramidal neurons. It was proposed that the decreased GABAergic inhibitory neurons excitability results in epilepsy in patients with DS (Marafiga et al., 2021). Moreover, NaV1.1 selective deletion in parvalbumin (PV)-positive interneurons or GABAergic interneurons of forebrain resulted in epilepsy in mice (Mantegazza et al., 2019). These findings are consistent with inhibitory neurons diminished excitability as a potential factor playing a role in seizures in patients of DS.

Additionally, by using homologous recombination two mouse models were generated carrying human mutations of SCN1A gene. First model had a mutation (R1407X) which results in the formation of truncated alpha subunit in DS patient (Isom et al., 2021). While second had a missense mutation (R1648H) identified in patients of GEFS (Das et al., 2021). Both mutations had similar effects in mouse i.e., diminished excitability of GABAergic inhibitory interneurons (Marafiga et al., 2021).

These results align with observations derived from mouse models of Scn1a, suggesting that SCN1A mutations result in suppression of inhibitory activity of neurons leading to seizures. These models also suggest that the underlying mechanism causing reduced inhibitory interneurons excitability is decrease in sodium current density (Chen et al., 2021).

1.10. Reported mutation of SCN1A

Most of the mutation in the SCN1A are missense, nonsense, splice-site and frame-shift mutations caused by base substitutions, deletions or insertion (Sun et al., 2010). Nonsense mutations (23%, 52/228), mutations altering splice sites (13%, 29/228), and small deletion or insertion leading to a frameshift (Depienne et al., 2008). Most of the mutations lies in the coding region (Exonic region), other lies in the noncoding (Intronic) and untranslated regions (UTR region). Most frequently mutated exons of SCN1A include exon 5, 15, 21, 25 and 26. Severe myoclonic epilepsy of infancy is an autosomal dominant disorder, according to one study in japan 8 mutations

were found in exon 26, 7 mutations were found in exon 15 and 2 mutations were found in exon 21 of SCN1A gene (Fakuma et al., 2004). In case of dravet syndrome, most mutations are reported in autosomal dominant and other in autosomal recessive pattern, according to one study 76 mutations were found in exon 26, 26 were found in exon 15 and 18 were found in exon 21 (Lal et al., 2016). According to study 13 mutations were found in patients having dravet syndrome with autosomal recessive pattern of inheritance in Pakistani population. Autosomal recessive mutation identified in SCN1A gene in other countries are listed below in table 1.2.

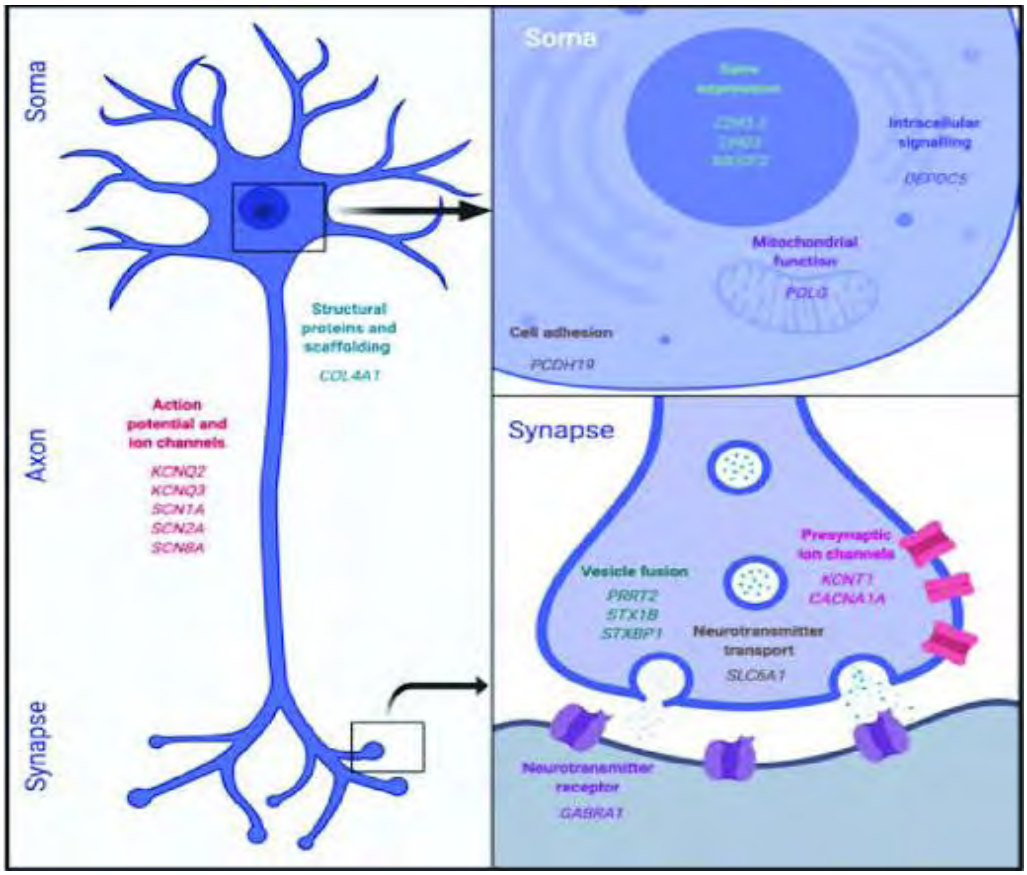

Figure 1.5. Neurotransmitter and Ion channels transporters, and problem in these can cause Epilepsy. Adopted from Rosch et al., (2019).

Table 1.2: A list of SCN1A mutations.

Phenotype	Inheritance	Gene	Area	Patients	Found in	Exon	Reference
SMEI	AD	SCN1A	Japan	58	8	26	Fukuma et al., 2004
				58	7	15	Fukuma et al., 2004
				58	2	21	Fukuma et al., 2004
Dravet/GFES+	AD			291	76	26	Lal et al., 2016
				291	26	15	Lal et al., 2016
				291	19	21	Lal et al., 2016
Familial epilepsy	AD/AR		Pakistan	216	13		Moller et al., 2016
Dravet/GFES+	AD/AR		Pakistan			Ts2298771 (G>A)	Ashfaq et al., 2022
Dravet/GFES+	AR		Germany			c.4513A>C	Marco Hernández AV et al., 2021
Dravet/GFES+	AR		Germany			c.302G>A	Marco Hernández AV et al., 2021
Dravet/GFES+	AR		Germany			C.2768T>G	Marco Hernández AV et al., 2021
Dravet/GFES+	AR		Germany			C.824A>G	Marco Hernández AV et al., 2021
Dravet/GFES+	AR		Germany			c.1198A>G	Marco Hernández AV et al., 2021
Dravet/GFES+	AR		Germany			c.1852C>T	Marco Hernández AV et al., 2021
	AR		Turkey			c.4796delA	Aslan et al., 2020
	AR		Turkey			c.1852C>T	Brunkla et al., 2015
	AR		Vietnam			c.4319C>T	Khanh et al., 2021
	AR		France			c.2768T>G	Moretti et al., 2021
	AR		Turkey			c.4727T>C +c.302G>A	Tuncer et al., 2015

1.11. Epilepsy Related Genes reported in Pakistan

The genetic landscape of epilepsy is not well defined in Pakistan, as very few studies have been reported. Besides, no systematic study has been conducted in Pakistan, so we don't have any epidemiological information on predominant genetic epilepsy type in Pakistan. Few studies have reported SCN1A, EFCH1, BRD2, and GABRG2 association with different types of epilepsies. Disease causing variants (e.g. c.2636T>C, c.2824C>G, c. 1376A>G) in SCN1A was found in 11 out of 216 patients (Moller et al., 2016). Mutations in EFCH1 gene was found in 19 out of 66 individuals in Pakistani population (Saleem et al., 2021). Mutations in BRD2 gene was found in 04 out of 14 individuals (Daud et al., 2022). Mutations in GABRG2 was identified in 43 out of 95 individuals (Iqbal et al., 2018).

Frequently mutated gene in individuals with Dravet syndrome in Pakistani population. In the majority of studies autosomal dominant pattern of inheritance is observed but few have also reported autosomal recessive pattern of inheritance. List of genes identified in Pakistan is provided in table 1.3.

Table 1.3: Epilepsy genes reported in Pakistan.

Phenotype	Inheritance	Gene	Area	Patients	Found in	Percentage	Reference
GFES+/Dravet syndrome	AD/AR	SCN1A	Pakistan	216	11	70-80%	Moller et al., 2016
JME	AR	EFCH1	Pakistan	66	19	10-30%	Saleem et al., 2021
JME	AR	BRD2	Baluchistan	14	04		Daud et al., 2022
CAE	AR	GABRG2	Punjab	95	43		Iqbal et al., 2018

Aim and objectives:

The present study aimed to recruit and perform genetic analysis on consanguineous families with Epilepsy with following objectives:

- To analyze clinical features of epilepsy patients in Pakistan.
- To identify genetic variants responsible for epilepsy in Pakistani population.

2. Materials and Methods

2.1. Ethical Approval

The research work was accepted by Bioethics committee of Quaid-I-Azam University Islamabad, Pakistan. For the current study, five families with epilepsy were recruited from different areas of Pakistan. Complete information of each family was collected by visiting these families in their native area and that information was used for the pedigree construction.

2.2. Family enrolment

Five families with epilepsy were recruited from different areas of Pakistan. These families were labeled as A, B, C, D and E.

2.3. Pedigree Construction

Pedigrees are helpful for finding the genetic relationship, number of patients with disease and their inheritance pattern. Pedigrees of five families were drawn by using Haplo-Painter software (https://haplopainter.soft112.com) (Holger & Nurnberg., 2005).The Genotypic and phenotypic information in form of tabular representation are known as Ped files, these files were drawn in Excel software and then entered into Haplo-painter. Generally, males were denoted by squares while females were represented by circle in pedigree. Unaffected and affected individuals were represented by filled and unfilled symbols, respectively.

The deceased individual was shown with diagonal line on their symbol. In pedigree double lines represented the consanguineous marriages while different generations were denoted by Roman numerals, while each person in the pedigree is represented with Arabic numeral.

2.4. Clinical Evaluation of Affected Individuals

Collection of information and evaluation of the affected individuals was done by visiting their residence. Each affected individual was analyzed to record clinical information like behavioral, morphological, skeletal, mental and neurological abnormalities. Severity of epilepsy was determined by seizure type and duration. Postnatal and prenatal history along with height, weight and head circumference (HC) was recorded for each affected individual of the five recruited families.

2.5. Blood Sample Collection

Peripheral blood sample of affected individuals was collected by using sterile syringes of 5-10ml. Blood was then preserved in vacutainer tube containing EDTA Ethylene di-amine tetra acetate) and were stored in lab of Genomics at 4°C.

2.6. Genomic DNA Isolation

Isolation of genomic DNA (gDNA) was carried out through organic (phenol-chloroform) method from peripheral blood samples (Sambrook & Russell., 2006). Brief composition of different solutions used during DNA extraction are mentioned in table 2.1. The protocol consists of three days which is as follow

2.6.1. Day 1

• From each individual 750µl of blood was taken in EDTA tubes and transferred to 1.5ml micro-centrifuge tubes (Gene Era Biotech Co.®, California, USA) followed by addition of 750µl solution A to each tube and incubated for 15 minutes at room temperature respectively. The tubes were inverted several time to speed up the lysis of cells.

• As the incubation time completed, the centrifugation was carried out for 1 minute at a speed of 12000rpm (revolution per minute) in a micro-centrifuge (Hettich Zentrifligen®, Mickro 120, Germany) which results in the formation of two layers, the upper one (supernatant) and the lower one (pellet). The supernatant was discarded in 10% bleach solution and the nuclear pellet was dissolved in 400µl of solution A. The

tubes were centrifuged again for 1 minute at a speed of 12000rpm that allow the DNA to settle down at the bottom of the tube.

• Again the supernatant was discarded and the pellet was redissolved by adding 400µl of solution B, 12µl of sodium dodecyl sulfate (20% SDS), and 5µl of proteinase-K (10 mg/ml). The tubes were then incubated in an incubator overnight (HeraThermTM Thermo Scientific, USA) at 37◦C.

2.6.2. Day 2

• On second day, solution C+D of equal volume was prepared that contain solution C (Saturated Phenol) and solution D (Isoamyl alcohol and chloroform) in 1:1 ratio. Then to the incubated tubes 500µl of solution C+D was added followed by centrifugation at 12000rpm for 10 minutes, which separated the mixture in two layers, the upper layer containing DNA was picked by pipette carefully to avoid its mixing with the lower layer and then shifted into a new micro-centrifuge tube and the lower layer was discarded.

• 500µl solution D was added to the tube, followed by centrifugation at 12000rpm for 10 minutes again resulted in two layers, the upper layer containing the DNA was shifted into a new micro-centrifuge tube and the lower layer was discarded. The DNA was precipitated out by adding 55µl of sodium acetate (3M, pH=6) and 500µl of chilled isopropanol and the tubes were inverted several times followed by centrifugation at 12000rpm for 10 minutes.

• After centrifugation the supernatant was discarded, and the pellet was washed with 200µl of 70% ethanol. Centrifugation was then carried out at 8000rpm for 7 minutes. The supernatant was poured out again and the tubes containing DNA pellets were kept at room temperature for 30 minutes to dry. After drying, the DNA pellets were dissolved in 80-150µl of Tris-EDTA (TE) buffer depending on the size of the pellet. The tubes were kept in an incubator for overnight incubation at 37◦C.

2.6.3. Day 3

• The tubes containing the desired DNA were taken out from incubator and stored at 20◦C. Further, the quality and quantity of DNA was checked as mentioned below:

2.6.4. Quantitative Analysis of DNA

Concentration of the DNA was determined by using Nanodrop (Colibri Titertek Berthold, Germany). The concentrations of DNA (ng/µl) were quantitatively analyzed along with the plotted graph, which showed the absorbance values (A260/A280) and (A260/A230) indicating the protein content, and other organic and inorganic contaminations respectively. The absorbance value in the range of 1.8-2.05 showed that the quality of DNA was good enough. Although concentration of DNA between 20-40ng/µl is considered the best-known DNA concentration for polymerase chain reaction (PCR). Dilutions were made by using the dilution formula in case concentration of any DNA sample higher than 50ng/µl.

$$C1V1=C2V2$$

2.6.5. Qualitative Analysis of DNA

The qualitative analysis of extracted DNA was done by using 1% agarose gel. The chemical composition of agarose gel is given in table 2.2.

• For gel electrophoresis, 1X tris borate EDTA (TBE) was used to prepare 1% agarose gel. The same buffer was used to run the DNA on the gel.

• A stock solution of TBE buffer (10X) was prepared and the gel was made through dilution of the stock solution with distilled water in 1:10.

• For 1% agarose gel preparation, 1g of agarose was weighted and added in 100 ml of 1X TBE in a conical flask. The solution was heated in a microwave for almost 2 minutes.

• As the solution cooled down, 10µl of ethidium bromide was added and mixed well. Now the agarose solution was poured into the casting tray having combs inserted in it. The gel was allowed to solidify for about 20-25 minutes.

• As the gel solidified, 1X TBE (1L) was added to the gel tank and after removing the combs, gel was transferred into it.

• 3µL of loading dye and 3µL of DNA from each sample (6X) was loaded in wells and run at 100-120 voltage for 60 minutes to analyze the DNA.

• Gel was visualized through Gel Doc System (INGENIUS SYNGENE Bio Imaging®, UK). The intensity and intactness of the bands on the gel are roughly proportional to DNA quality and quantity respectively.

2.7. Candidate Gene Selection

Genes were chosen for each family by a systematic review of the literature led to the listing of genes involved in epilepsy in Pakistani patients and worldwide. The genes with the most relevance to the phenotypes of understudies. Families involved in our study were selected for screening in five families.

2.8. Primer Designing

Primers are 18-24bp oligonucleotides which are used to amplify specific regions of the DNA through PCR. Various bioinformatics tools and browsers were used to design primers for selective exons of each gene.

2.8.1. Ensemble Genome Browser

Firstly, the sequences of ***SCN1A*** gene (NM_001202435) in FASTA format was downloaded from the Ensemble Genome Browser by selecting the longest transcript with specific NMID. The list of all selected primers used in the current study were designed by the following software as discussed below and list of these primers are given in table 2.3.

2.8.2. Primer 3 Input

Primer3 input browser (https://primer3.ut.ee/) was used to design the primers of the selected exon by choosing specific conditions like Primer length (18 – 22), GC Content (40-60%), Tm (56-64◦C) and Tm difference (<1◦C) (Untergasser et al., 2012).

2.8.3. In Silico PCR

This software (https://genome.ucsc.edu/cgi-bin/hgPcr) provided information about the size of a hypothetical amplicon. Sensitivity, efficiency, and performance of different primers under optimum temperature was evaluated. Various in silico tools were used to evaluate the quality of primers as discussed below:

2.8.4. Human Blat Search UCSC Genome Browser

This software (https://genome.ucsc.edu/cgi-bin/hgBlat) was used to confirm the specificity of primers that whether each primer had single or multiple hits in the genome and primers with single hit were selected.

2.8.5. Oligo Calc

This software (http://biotools.nubic.northwestern.edu/OligoCalc.html) was used to investigate various properties of designed primers like hairpin loop formation, self-complementarity, and mismatches like the binding of primer at any other place than selective exon through 3'end complementarity.

2.9. PCR

PCR, used to amplify the small size of DNA fragment, comprising of three steps i.e. denaturation, annealing and extension (Mullis and Faloona, 1987). Thermocycler (Biometra®, Germany) was used to carry out the PCR. After preparing and adding the reagents, the PCR tubes were vortexed following the short spin. These PCR tubes were shifted in the thermocycler (Biometra®, Germany) under specific annealing conditions for each primer and extension time required for desired product formation. One primers set of ***SCN1A Exon 5,15,21 and 25*** were designed but for exon 26 two overlapping primer sets were designed to amplify by using open reagent PCR. The PCR reagents composition and profile is given in table 2.4 and 2.5 respectively.

2.9.1. Agarose Gel Electrophoresis

The estimated size and quality of the PCR product was checked by visualizing the bands in the Gel Doc System (INGENIUS SYNGENE Bio Imaging®, UK) after running it on 2% agarose gel at voltage of 120V for 20-25 minutes. The image was saved in the connected database with Gel Doc System. The chemical composition of 2% gel is given in table 2.2.

2.9.2. PCR Product Purification

PCR purified product was required for the applications like sanger sequencing. The non-specific products like dNTPs, primer dimers and enzymes were removed by using different protocols discussed below:

2.9.2.1. EXOSAP-ITTM Purification

Equal volume of ExoSAP reagent and water were mixed for each reaction. 2µl of EXOSAP IT reagents (Thermo Scientific) from mixture and 6µl of PCR product were added and tubes were placed in thermocycler (Biometra®, Germany). The selected profile consists of 15-minute incubation at 37°C which hydrolyze the excessive primers and nucleotides in a single enzymatic reaction followed by 15-minute incubation at 80°C to inactivate the ExoSAP-IT reagent. After this procedure of 30 minutes, the bands of purified products were examined on the 2% agarose gel under UV light. Bright and clear bands confirmed the quality of purified product for sanger sequencing.

2.9.2.2. Gel Purification

The nonspecific products were present in the amplicon of exon 26A and 26B of *SCN1A*. Gene Jet Purification Kit (Thermo Scientific, USA) was used to purify these amplicons. The procedure is as follow:

• 1.5 ml micro-centrifuge tubes were pre-weighed and sterilized blade was used to cut the gel containing desired PCR product. The difference between the pre-weight and post-weight readings was then used to calculate the weight of the gel slice. The gel mixture was combined with an equal volume of binding buffer (1:1) and incubated at

60 °C in the incubator until the gel slice was fully dissolved. The tube was inverted several times.

• The tubes were inverted several times, and the gel mixture was transferred on the Gene JET purification column and centrifuge for 1 minute at 8000rpm. The column was put back into the same collection tube after the flow-through was discarded. 100µL of binding buffer was added to the column and centrifuged it for 1 min. The column was kept in the same collection tube again after discarding the flow through. 700µL of wash buffer (diluted with ethanol) was added to the column and centrifuged for 1 min. Impurities from the walls of column were removed via repeated centrifugation.

• Column was repositioned into new 1.5ml micro-centrifuge tube. Elution buffer was added to the column depending upon the volume required followed by centrifugation for 1 minute. The DNA obtained in flow through was qualitatively analyzed by running it on 2% agarose gel. Clear and bright bands according to estimated size confirmed the quality of purified product for sequencing.

2.10. Gene Sequencing

Purified products of PCR with clear bands were further proceeded for Sanger sequencing. 4µl of Terminator Ready Reaction Mix, 4µl of DNA template (30-90ng), 1µl of primer and 1µl of ddH2O were added in 0.2ml of micro-map tubes followed by short spin. Tubes were placed in the thermocycler and profile was set comprising of 25 cycles with incubation 96°C for 10 seconds, then 50°C for 10 seconds and 60°C for 4 minutes, and stored at 4°C.

40µL (75%) and 30µL (100%) of isopropanol were mixed in a 1.5ml of micro-centrifuge tube in order to purify the sequencing products followed by incubation of 15 minutes at room temperature. The samples were centrifuged, and supernatant were discarded. 75% isopropanol was used to wash DNA pellet followed by centrifugation for five minutes. Again supernatant was discarded and the sample was placed in a vacuum centrifuge tube for 10-15 minutes and stored at 20°C. The pellet of the sample

was dissolved in 3µL of loading buffer, centrifuged, and heated at 95°C for 2-3 minutes. Finally, tubes were put on chilled ice, and the purified product was sequenced.

2.11. In Silico Analysis of Sequencing Data

2.11.1. Bio Edit Software

The sequencing results were analyzed through the **Bio-edit Software** (Hall, 1999) by aligning the sequence results with consensus sequence of specific exon downloaded from the Ensemble Genome Browser using the ClustalW Alignment Option (Thompson et al., 1994). Any changes in the nucleotide and chromatogram were examined properly. The variants identified in data were further investigated through different bioinformatics tools.

2.11.2. Mutation Taster

This tool predicts about the pathogenic nature of the variant that either it is disease causing or not (Schwarz et al., 2014). It gives us the PhyloP and PhastCons scores that help us to determine the grade of conservation of given nucleotides. The phastCons values lie within range of 0 to 1 (the closer the value is to one, the more likely the nucleotide is conserved, and the variation is pathogenic). PhyloP scores range from -14 to +6. In comparison to a negative score, a positive score indicates slower evolution than expected under neutral drift, but also acceleration. (Siepel et al., 2005).

2.11.3. UMD Predictor

It predicts the pathogenicity of single nucleotide variants (SNVs) by generating a score. The pathogenicity of more than one variant can be predicted by this tool in a single run. A score of 100 for a variant is considered highly pathogenic.

2.11.4. Combined Annotation Dependent Depletion (CADD)

The harmfulness of SNVs including indels in the human genome was examined using CADD. Variant with positive CADD scores is deleterious (D), whereas one with negative score indicate that it is neutral (Rentzsch et al., 2019).

2.11.5. Varsome

Varsome is a useful annotation tool and search engine for finding human genome variations. It helps us to classify the mutation as 'pathogenic', 'likely pathogenic', 'benign', or 'uncertain relevance' by using standards of American College of Medical Genetics and Genomics (Kopanos et al., 2019).

2.11.6. Splice AI

This web tool allows us to look for the variants that can influence splicing in the human genome. It predicts the effect of the variant on the splice donor and acceptor site.

2.11.7. Franklin

It is a variant interpretation tool. It classifies variants based on ACMG and AMP guidelines. It gives weights to various in-silico tools and generates an aggregated prediction in the form of a score.

Table 2.1: Chemical composition and concentrations of DNA extraction stock solutions and buffers

Stock Solution	Chemical composition	Concentrations
Solution A	Sucrose	0.32M
	Tris HCl (pH= 7.5)	10mM
	Magnesium Chloride ($MgCl_2$)	5mM
	Triton X-100 (v/v)	1%
Solution B	Tris HCl (pH= 7.5)	10mM
	Sodium Chloride (NaCl)	400mM
	EDTA (pH= 8)	2mM
Solution C	Saturated Phenol	
Solution D	Chloroform	24 volumes
	Isoamyl alcohol	1 volume
20% SDS	SDS	10g
	Water	50mL
Loading Dye	Sucrose	40g
	Bromophenol	0.25g
10X TBE	Tris	0.89M
	Boric Acid	0.89M
	EDTA (pH= 8)	0.89M

Table 2.2: Composition of agarose gel electrophoresis

Sr. No.	Reagents	Volume/Reaction
1.	Agarose	1 – 2 %
2.	1X Tris-EDTA-Boric acid (TBE) Buffer	100ml
3.	Ethidium Bromide	Upto 10ml

Table 2.3: List of *SCN1A* primers

Primer Name	Primer Sequences	Tm (°C)	Product Size (bp)
SCN1A Ex-5F	AGGGCTACGTTTCATTTGTA	55	473
SCN1A Ex-5R	TCAACTACTGCAAAGCCTTC	56	
SCN1A Ex-15F	TTACCTGGGCTCTATGTGTG	56	715
SCN1A Ex-15R	TGAGCTACTTCTGGATCCTG	56	
SCN1A Ex-21F	GCCTTAAATTCAACAGCAACT	56	662
SCN1A Ex-21R	GAGGCCTATTTCTCTTGCAT	56	
SCN1A Ex-25F	TACCTGGGCTCTATGTGTGT	56	714
SCN1A Ex-25R	TGAGCTACTTCTGGATCCTGA	58	
SCN1A Ex-26AF	TACTGTTCGGCCAAATGTGC	62	935
SCN1A Ex-26AR	ACTGAGTTGCATCGGGATCA	62	
SCN1A Ex-26BF	CTGAAGAAAGTGCAGAGCC	56	849
SCN1A Ex-26BR	GGTCATCTCCCCTTTACACA	57	

Table 2.4: Polymerase Chain Reaction reagents (open reagent)

Sr. No.	PCR Reagents	Volume Per Reaction
1	10X Taq Buffer (NH4)2SO4 or 10X Taq Buffer KCl (ThermoScientific)	2.5µl
2	MgCl2 (Thermo Scientific)	0.8-1µl
3	dNTPs (10mM, Thermo Scientific)	0.5µl
4	Taq. Polymerase (Thermo Scientific)	0.2µl
5	Template DNA (50-100ng)	1µl
6	Forward Primer (10µM)	1µl
7	Reverse Primer (10µM)	1µl
8	Nuclease free water	up to 25µl

Table 2.5: General PCR profile

Steps	**Cycles**	**Temperature (°C)**	**Time Duration**
Initial Denaturation		95.0	10 min
Denaturation	40 Cycles	95.0	1 min
Annealing		58.0-64.0	1-1.5 min
Extension		72.0	1-2 min
Final Extension		72.0	10 min
Pause		4.0	∞

3. Results

3.1. Clinical Profile of Families

Five families (Family A, B, C, D and E) having epilepsy were recruited from the different areas of Pakistan. Each family had more than 1 individual affected with epilepsy. Clinical features of the affected members of these families were collected and analyzed to see similarities. Detailed clinical profile of these patients is discussed below.

3.1.1. Family A

The pedigree of family A depicted autosomal recessive pattern of inheritance (Figure 3.1). Four generations are shown in the pedigree. This family has 3 children including, 1 normal girl (IV:1) and 2 affected individuals, 1 male (IV:2) and 1 female (IV:3) of age 13 and 7 years respectively born to a consanguineous Pakistani family. Blood was taken from all the family members. The 13 years old male patient (IV:2) had its first fits at the age of 5 months while the female patient (IV:3) at age of 9 months. Both patients had their first seizure with fever and were diagnosed with Tonic clonic seizures.

Both patients had normal birth. Both showed intellectual disability, developmental delay, aggressive behavior, speech anomaly and abnormal sleep. Clinical features of family A are listed in the table 3.1.

3.1.2. Family B

The pedigree of family B depicted autosomal recessive pattern of inheritance (Figure 3.2). Four generations are shown in the pedigree. This family has 6 children (boys) including, 4 normal and 2 affected individuals of age 9 and 8 years, respectively. Blood was taken from all family members. The 9 years old male (IV:4) had its first fits at the age of 6 months while, the patient (IV:6) at the age of 11 months. Patient (IV:4) had its first seizure with fever and both patients were diagnosed with Tonic clonic seizures.

Both patients have a history of birth through a cesarean section. Both showed intellectual disability, imbalance posture, aggressive behavior, and speech anomaly. Clinical features of family B are listed in the table 3.2.

3.1.3. Family C

The pedigree of family C depicted autosomal recessive pattern of inheritance (Figure 3.3). This family has 3 children, 1 normal female (IV:1) and 2 affected females, (IV:2) and (V:3) of age 8 and 6 years respectively born to a consanguineous Pakistani family. Blood was taken from all the family members. The 8 years old female (IV:2) had its first fits at the age of 3 months while the patient (IV:3) at age of 7 months. The patient (IV:3) is completely on bed. The patient (IV:2) had its first seizure with fever, was diagnosed with febrile seizures and patient (IV:3) was diagnosed with Tonic Clonic seizures.

Both patients were delivered through a cesarean section. Both showed intellectual disability, developmental delay, skeletal abnormality, imbalance posture, aggressive behavior, and sleep problems, while patient (IV:3) has speech anomaly. Clinical features of family C are listed in the table 3.3.

3.1.4. Family D

The pedigree of family D depicted autosomal recessive pattern of inheritance (Figure 3.4). This family has 6 children, 3 females and 3 males, 3 normal boys, 1 girl and 2 affected females, (V:1) and (V:2) of age 13 and 3 years respectively born to a consanguineous Pakistani family. Blood was taken from all the family members. The 13 years old patient (V:1) had its first fits at the age of 3 months while the patient (V:2) at age of 11 months. The patient (V:1) had his first seizure with fever and both patients were diagnosed with Tonic clonic seizures.

Both patients had normal birth. The individual (V:1) showed intellectual disability, aggressive behavior, skeletal abnormality, speech anomaly, abnormal posture and abnormal sleep. Clinical features of family D are listed in the table 3.4.

3.1.5. Family E

The pedigree of family E depicted autosomal recessive pattern of inheritance (Figure 3.5). Four generations are shown in the pedigree. This family has 3 children. 1 normal girl (IV:3) and 2 affected male individuals, (IV:1) and (IV:2) of age 14 and 12 years respectively born to a consanguineous Pakistani family. Blood was taken from all the family members. The 14 years old individual (IV:1) had its first fits at the age of 9 months while (IV:2) at age of 1 year. Both patients had their first seizure with fever and have Tonic clonic seizures.

Both patients had normal birth. The individual (IV:2) showed aggressive behavior. Clinical features of family E are listed in table 3.5. Both patients had their first seizure with fever, and both were diagnosed with Tonic clonic seizures.

3.2. Candidate Gene Analysis

The systematic literature review was carried out to identify the genes that were previously associated with inherited epilepsy. Mutations in various genes which are involved in maintaining the proper functioning of the brain have been reported. Till now, 168 genes have been reported to link with pure epilepsy, out of which mutations in *SCN1A* are more frequent (70-80%) and correlated with the phenotype (Noebels et al., 2014). A total of 1966 SCN1A mutations have been reported on ClinVar. Out of these 688 are pathogenic, 332 are likely pathogenic, 652 of uncertain significance, 364 are likely benign and 129 are benign. Most of the pathogenic variants lie in exon 5 (8%),15 (10%),21 (15%),25 (20%), and 26 (40%) of SCN1A gene (Variants in gene SCN1A - ClinVar Miner (utah.edu)). Therefore, these exons were screened out.

3.2.1. Family A

The sanger sequencing of the selected exons was performed in (IV:2) individual; however, no variant was identified in this family.

3.2.2. Family B

The sanger sequencing of the selected exons was performed in (IV:4) individual, which resulted in the identification of two homozygous variants i.e., g.19189-19190insG and g.82579A>G.

The physical location of g.19189-19190insG is chr2:166910960-166910961insC (hg19) and lies in intron 5 of SCN1A (Figure 3.6). Mutation taster, and UMD predictor (score:00), predicted it to be polymorphism. PhyloP and PhastCons scores are -0.16 and 0, respectively. Results of Splice Al showed that this specific variant has no impact on splicing. According to ACMG and AMP classification it is also predicted as benign. Both variants are not reported in TGP or ExAC.

The physical location of g.82579A>G is chr2: 166847571T>C (hg19) and lies in untranslated region (UTR) of exon 26 (Figure 3.7). CADD score of g.82579A>G is 14.6, Raw score is 1.45 indicating the effect of this substitution is not deleterious. Mutation taster, and UMD predictor, predicted it to be disease causing. PhyloP and PhastCons scores are 0.93 and 0.92, respectively. Moreover, results of Splice Al and Pangolin (score:0.02) showed that variant can influences splice donor site which is almost 57 bp away from g.82579A>G variant. According to ACMG and AMP classification it is also predicted as benign.

Though two variants were identified in family B,but none of these criteria of being pathogenic.

3.2.3. Family C

The sanger sequencing of the selected exons was performed in (IV:2) individual; however, no variant was identified in this family.

3.2.4. Family D

The sanger sequencing of the selected exons was performed in (V:1) individual, which resulted in the identification of two homozygous variants i.e., g.64092_64093isnA and g.82579A>G.

The physical location of g.64092_64093isnA is chr2: 166866057_166866058insT (hg19) and lies in intron 21 (Figure 3.8). Mutation taster, and UMD predictor (score:00), predicted it to be polymorphism. PhyloP and PhastCons scores are 1.445 and 0.916 respectively. Results of Splice Al (score: 0.02) and Pangolin (score: 0.03) showed that this specific variant has impact on splice donor site. According to ACMG and AMP classification it is also predicted as benign. Both variants are not reported in TGP or ExAC.

The variant g.82579A>G is the same variant as identified in the family B (Figure 3.9), already discussed in section 3.2.2. Though two variants were identified in family B, but none of these criteria of being pathogenic.

3.2.5. Family E

The sanger sequencing of the selected exons was performed in (IV:1) individual; however, no variant was identified in this family.

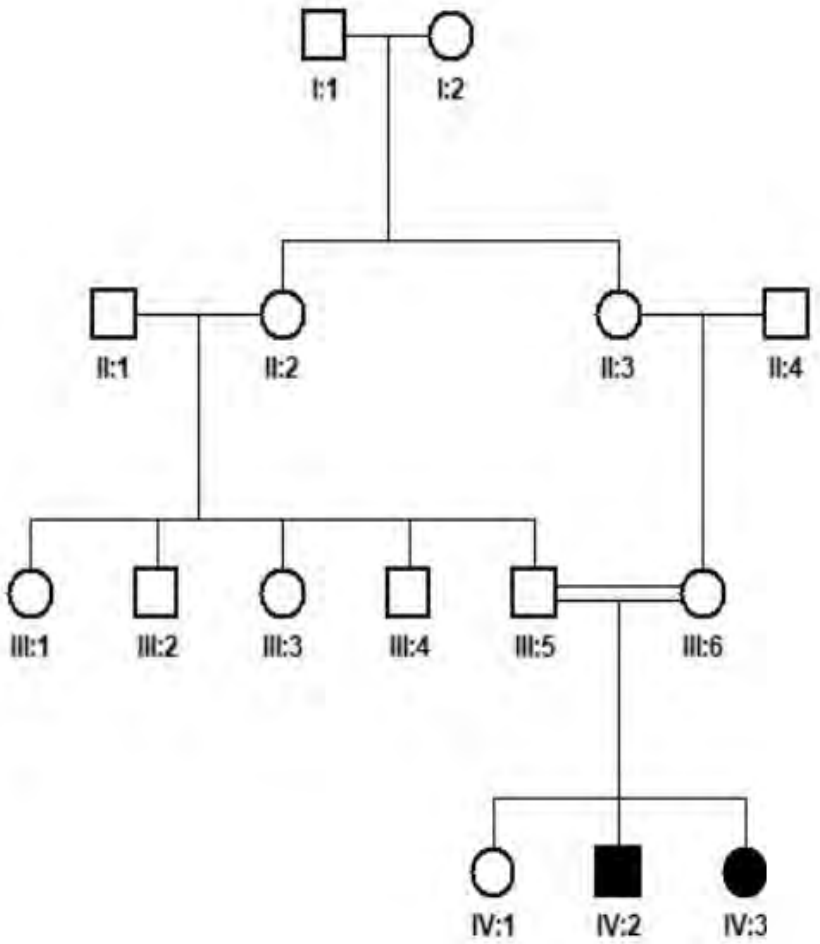

Figure 3.1. Pedigree of family A, designed by haplopainter, showing the autosomal recessive pattern of inheritance.

Table 3.1: Clinical profile of family A

Patient ID	**IV:2 (proband)**	IV:3
Gender	Male	Female
Age of onset	5 months	9 months
Age (Current)	13 years	7 years
Weight	18 kg	12 kg
Height	4 feet	3.7 feet
Microcephaly	No	No
Intellectual disability	Yes	Yes
Posture	Imbalance posture	Imbalance posture
Head circumference	54 cm	51.5 cm
Skin	Normal	Normal
Skeletal abnormality	No	No
Self-care	No	No
Aggressive behavior	Yes	Yes
Mood instability	Normal	Normal
Developmental delay	Slow growth	Slow growth
Delivery event	Normal	Normal
Speech anomaly	Abnormal	Abnormal
Sleep	Abnormal	Abnormal
Seizure	First seizure with fever	First seizure with fever
Seizure type	Tonic clonic	Tonic clonic

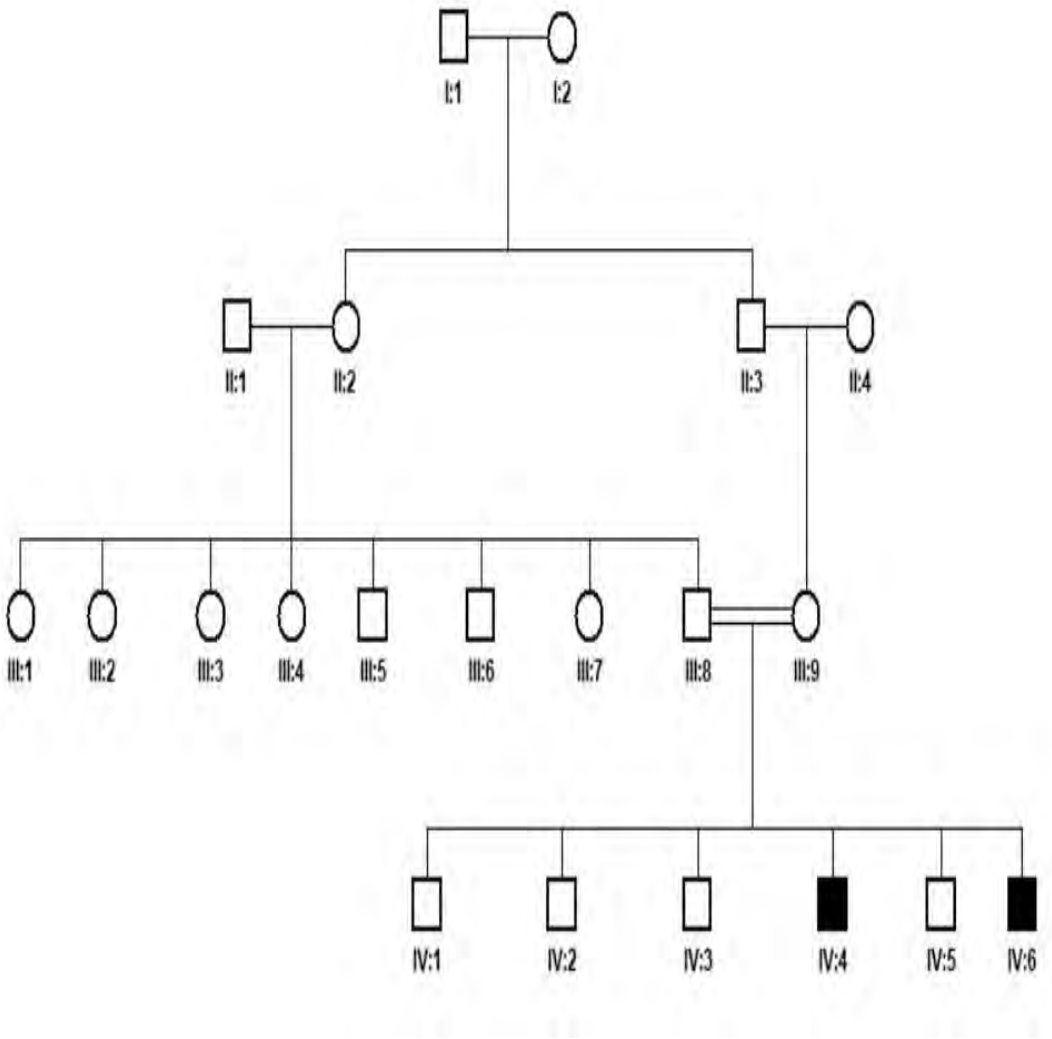

Figure 3.2. Pedigree of family B, designed by haplopainter, showing the autosomal recessive pattern of inheritance.

Table 3.2: Clinical profile of family B

Patient ID	**IV:4 (proband)**	IV:6
Gender	Male	Male
Age of onset	6 months	11 months
Age (Current)	9 years	8 years
Weight	21 kg	21 kg
Height	4 feet	4 feet
Microcephaly	No	No
Intellectual disability	Yes	Yes
Posture	cannot walk and eat	Cannot walk and eat
Head circumference	52 cm	52 cm
Skin	Normal	Normal
Skeletal abnormality	No	No
Self-care	No	No
Aggressive behavior	Yes	Yes
Mood instability	Normal	Normal
Developmental delay	Normal growth	Normal growth
Delivery event	C- section	C-section
Speech anomaly	Abnormal	Abnormal
Sleep	Normal	Normal
Seizure	First seizure with fever for 5 days	Seizure without fever
Seizure type	Tonic clonic	Tonic clonic

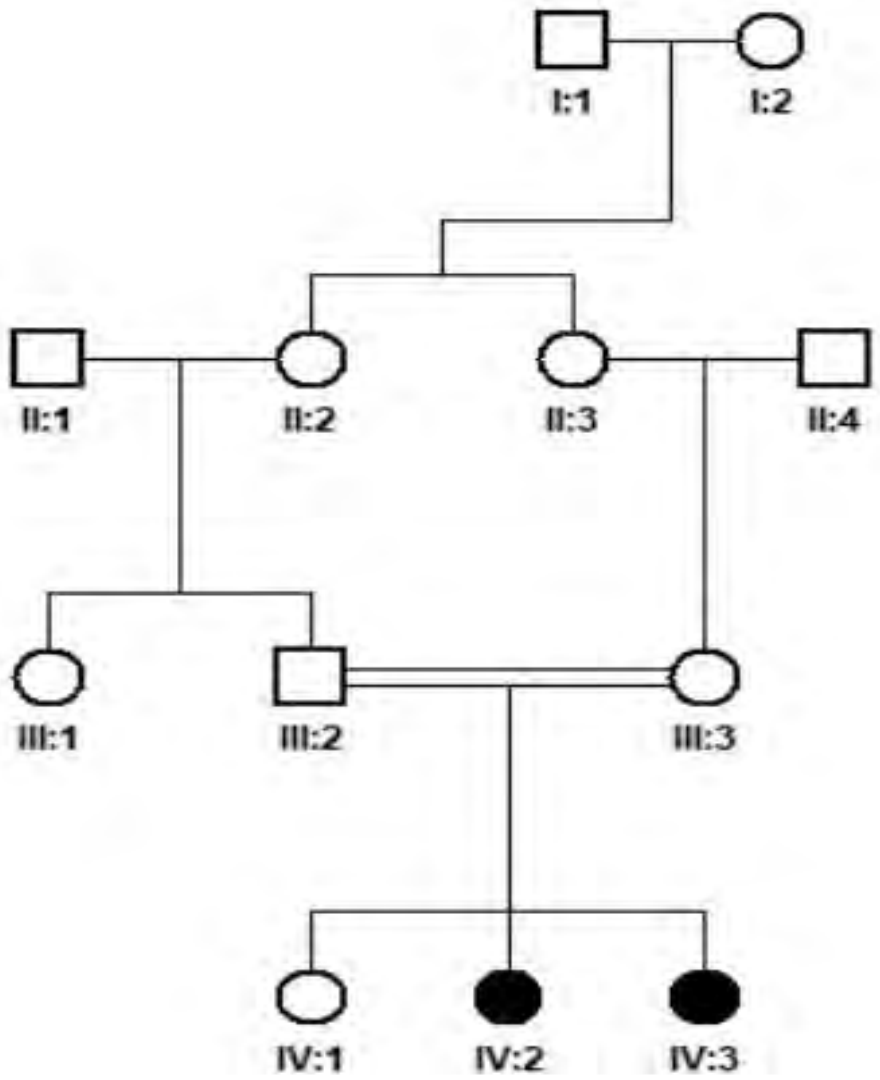

Figure 3.3. Pedigree of family C, designed by haplopainter, showing the autosomal recessive pattern of inheritance.

Table 3.3: Clinical profile of family C

Patient ID	**IV:2 (proband)**	IV:3
Gender	female	Female
Age of onset	3 months	7 months
Age (Current)	8 years	6 years
Weight	22 kg	17 kg
Height	4.1 feet	3.5 feet
Microcephaly	No	No
Intellectual disability	Yes	Yes
Posture	Imbalance posture	Imbalance posture
Head circumference	51.5 cm	50.5 cm
Skin	Normal	Normal
Skeletal abnormality	Yes	Yes
Self-Care	No	No
Aggressive behavior	No	No
Mood instability	Normal	Normal
Developmental delay	Slow growth	Slow growth
Delivery event	C-section	C-section
Speech anomaly	Normal	Cannot speak
Sleep	Abnormal	Abnormal
Seizure	First seizure without fever	First seizure with fever
Seizure type	Febrile seizure	Tonic clonic seizure

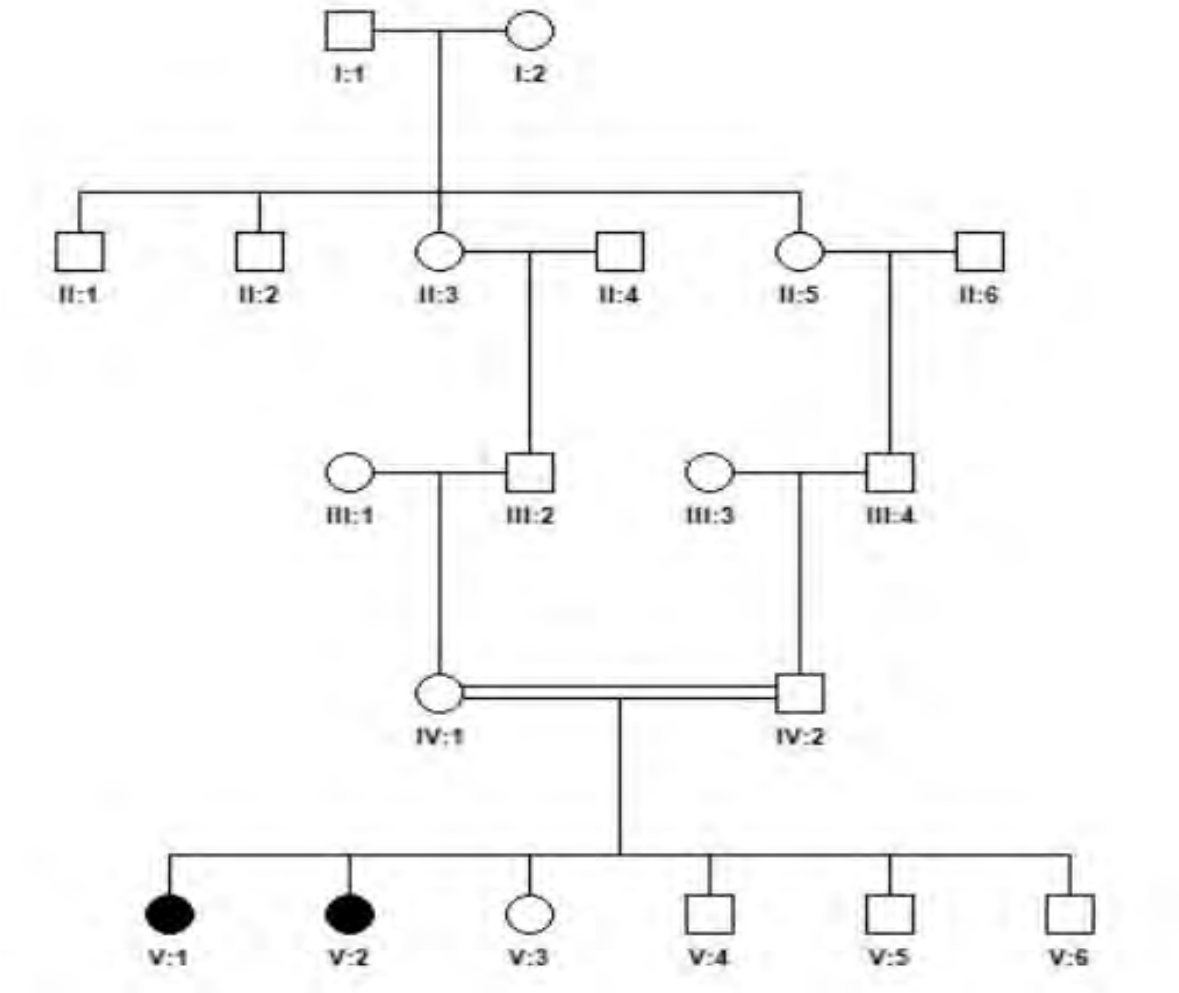

Figure 3.4. Pedigree of family D, designed by haplopainter, showing the autosomal recessive pattern of inheritance.

Table 3.4: Clinical profile of family D

Patient Id	**V:1 (Proband)**	V:2
Gender	Female	Female
Age of onset	3 months (15 days regular)	11 months
Age (Current)	13 years	7 years
Weight	20 kg	14 kg
Height	4.5 feet	3 feet
Microcephaly	No	No
Intellectually disable	Yes	No
Posture	Cannot walk and stand	Can walk properly
Head circumference	53 cm	51 cm
Skin	Normal	Normal
Skeletal abnormality	Yes	No
Self-Care	No	No
Aggressive behavior	Yes	No
Mood instability	Normal	Normal
Developmental delay	Normal	Normal
Delivery event	Normal	Normal
Speech anomaly	Abnormal	Normal
Sleep	Normal	Abnormal (can sleep for 2 days)
Seizure	First seizure with fever	First seizure without fever
Seizure type	Tonic-clonic seizure	Tonic- clonic seizure

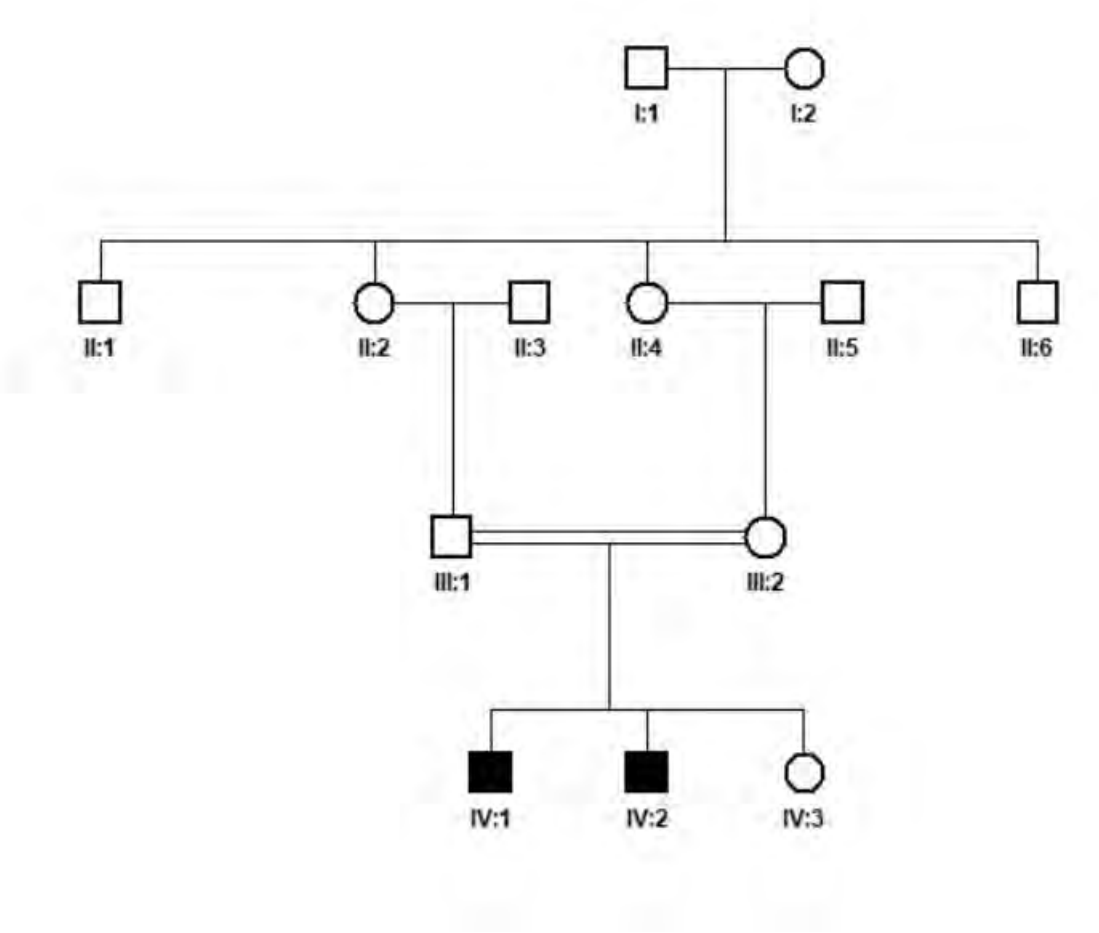

Figure 3.5. Pedigree of family E, designed by haplopainter, showing the autosomal recessive pattern of inheritance.

Table 3.5: Clinical profile of family E

Patient ID	**IV:1 (proband)**	IV:2
Gender	Male	Male
Age of onset	Less than 1 year	Less than 1 year
Age (Current)	14 years	12 years
Weight	22 kg	16 kg
Height	5.5 feet	4.7 feet
Microcephaly	No	No
Intellectually disable	Yes	Yes
Posture	Normal	Normal
Head circumference	54 cm	54 cm
Skin	Normal	Normal
Skeletal abnormality	No	No
Self-Care	Yes	Yes
Aggressive behavior	Yes	No
Mood instability	Normal	Normal
Developmental delay	Normal	Normal
Delivery event	Normal	Normal
Speech anomaly	No	No
Sleep	Normal	Normal
Seizure	First seizure with fever	First seizure with fever
Seizure type	Tonic clonic	Tonic clonic

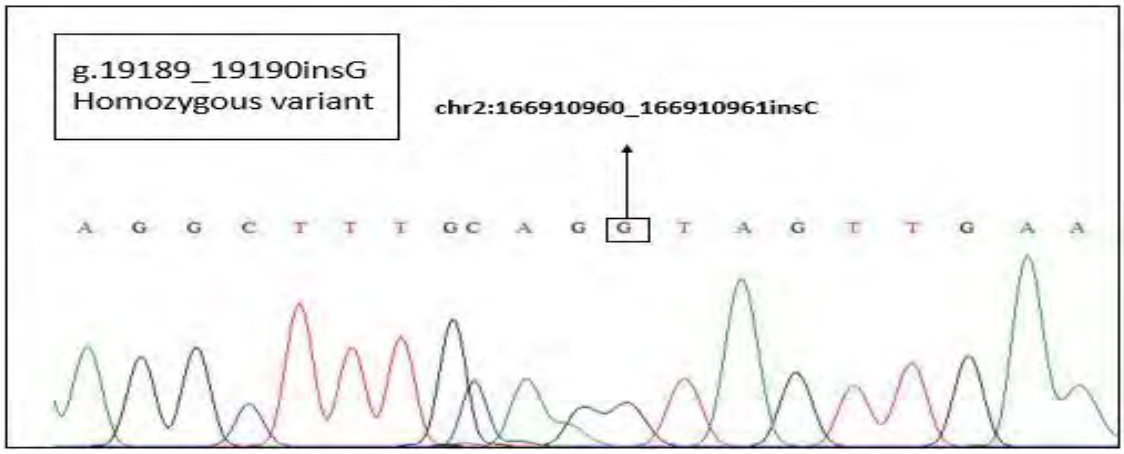

Figure 3.6: Sequencing chromatogram showing homozygous nucleotide variant g.82579A>G in the affected individual (IV:4) of family B.

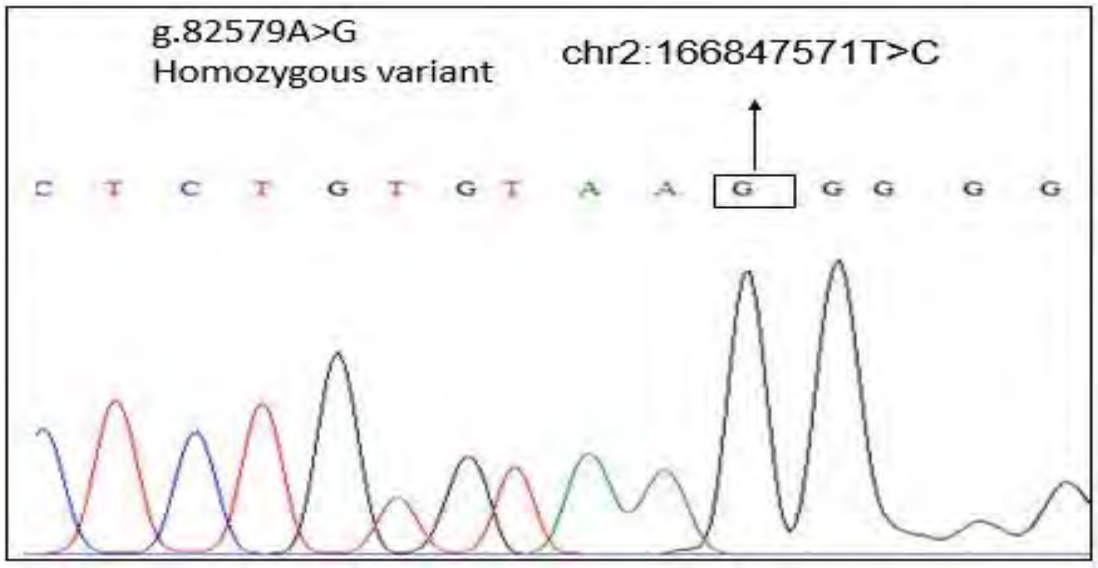

Figure 3.7: Sequencing chromatogram showing homozygous nucleotide variant g.82579A>G in the affected individual (IV:4) of family B.

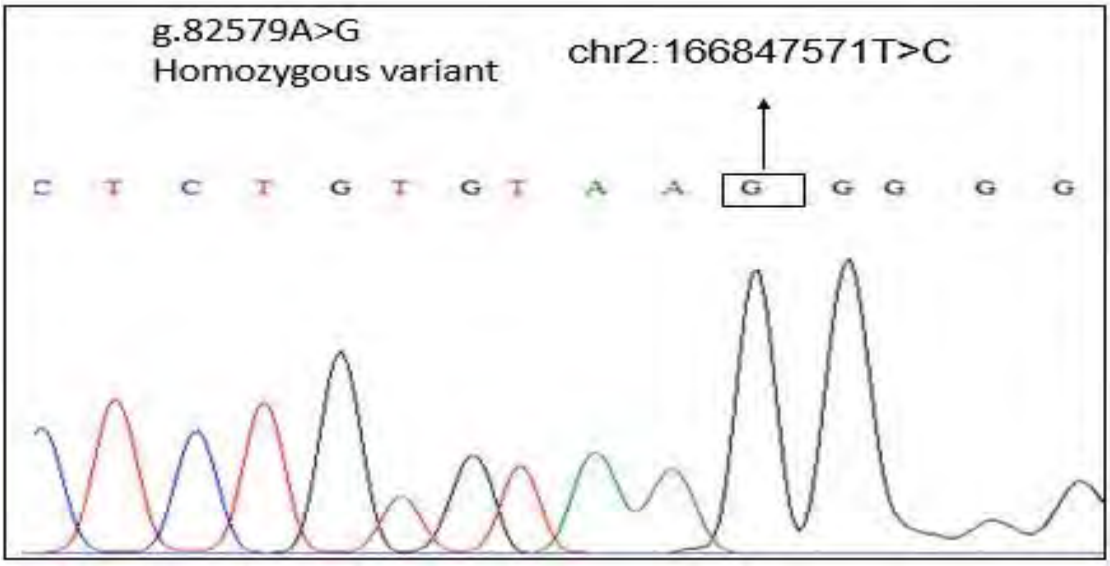

Figure 3.8: Sequencing chromatogram showing homozygous nucleotide variant g.82579A>G in the affected individual (V:1) of family D.

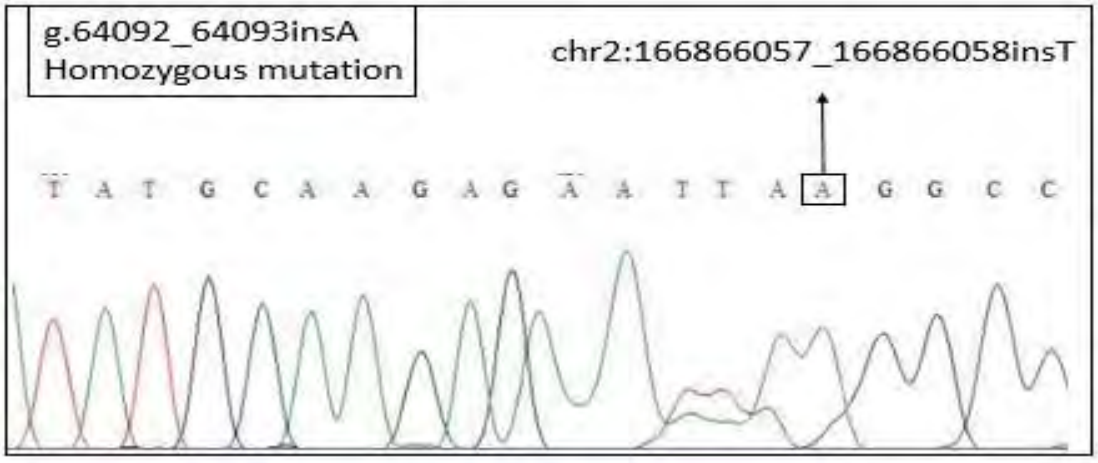

Figure 3.9: Sequencing chromatogram showing homozygous nucleotide variant g.64092_64093isnA in the affected individual (V:1) of family D.

Table 3.6: In silico analysis of variants identified in family A, B, C, D and E.

Family name	**B**	**B**	**D**	**D**
Individual ID	IV:4	IV:4	V:1	V:1
Gene name	SCN1A	SCN1A	SCN1A	SCN1A
Exon	26	05	21	26
DNA position	chr2: 66847571T>C	chr2:166910960-16691096insC	chr2: 166866057_166866058insT	chr2: 66847571T>C
Genomic position	g.82579A>G	g.19189-19190insG	g.64092_64093isnA	g.82579A>G
Protein Change	-	-	-	-
Zygosity	Homozygous	Homozygous	Homozygous	Homozygous
Previously reported	Nil	Nil	Nil	Nil
Reference ID	-	-	-	-
Mutation Taster	Disease causing	polymorphism	polymorphism	Disease causing
CADD	14.7	-	-	14.7

4. Discussion

Epilepsy (OMIM # 613060) is a disorder which is characterized by recurrent seizures. Epilepsy is a heterogeneous disorder, which results from interaction between complex genes and environmental factors (Steinlein et al., 2008).

Total number of genes reported for epilepsy without any neurodevelopmental or structural disorder are 168. Out of these 90 genes (53.6%) show autosomal dominant inheritance and 62 genes (36.9%) show autosomal recessive inheritance (Zhang et al.,2023). However, mutations in a few genes are reported for both autosomal dominant and recessive types of epilepsy including SCN1A (Lal et al., 2016; Marco et al., 2021), SCN2A (Corbett et al., 2016 and AlSaif et al., 2019), KCNQ2 (Perucca et al., 2019; Kuersten et al., 2020), KCNQ3 (Allen et al., 2014; Lauritano et al., 2019), STXBP1 (Khaikin et al., 2016; Balagura et al., 2022), PRRT2 (Zhao et., 2020; Musa et al., 2023), SLC2A1 (Klepper et al., 2009; Algahtani et., 2020) and DEPDC5 (Picard et al., 2014; Myers et al., 2017). Phenotype heterogeneity has been observed in various genes. Out of most reported genes for epilepsy, SCN1A (OMIM#182389) alone accounts for 70-80% of cases (Gambardella et al. (2009). Additionally, EFCH1 is involved in 10-30% cases (Saleem et al., 2021), while other genes SCN2A, STXBP1, SCN8A are reported in few families (Steel et al., 2017).

The reported genes of epilepsy play an important role in cellular pathways such as apoptotic and necrotic cell death pathways (Akyuz et al., 2021a). and receptor activation pathways (Akyuz et al., 2021b).

Though more than 30 genes have been identified for autosomal recessive epilepsy Calhoun et al., (2018), but literature review showed that SCN1A gene is a commonly mutated gene (Choohan et al., 2022). Mutations in the SCN1A gene are reported to cause both autosomal dominant and autosomal recessive epilepsy e.g. Dravet syndrome. This gene plays a vital role in controlling the flow of sodium (Na) into the neurons (Ademuwagun et al., 2021). Typically, interneurons play a role in inhibiting the generation of electrical activity in brain cells (Marafiga et al., 2021). However, when SCN1A variant is present, interneurons lose their ability to suppress electrical activity,

ultimately continuous firing of neurons, which causes electrical imbalance in brain cells leading to seizures (Bryson et al., 2021). Functional studies on mouse models have also demonstrated reduced excitability in inhibitory interneurons because of deletion of SCN1A gene's last exon (Meisler et al., 2021). This study has concentrated on the investigation of SCN1A mutations responsible for causing epilepsy in five families.

Most of the currently identified mutations in the SCN1A are missense, nonsense, splice-site and frameshift in nature (Sun et al., 2010). Nonsense mutations (23%), mutations altering splice sites (13%), and small deletion or insertion leading to a frameshift Most of the mutations lies in the coding region (Exonic region), but other lies in the noncoding (Intronic) and untranslated regions (UTR region) (Depienne et al., 2008).

In the current study five families A, B, C, D and E were recruited from different areas of Pakistan. Clinical investigation of affected individuals from these families showed, developmental delay, moderate to severe intellectual disability, Seizures, mood instability, and speech anomaly. In addition, skeletal abnormality (except family A,B, and E), sleep abnormality (except family B), aggressive behavior (except family C) and absence of self-care abilities (except family C and E) were noted in affected individual of these families.

The clinical features of the affected individuals align with Dravet syndrome. Considering the high prevalence of SCN1A in the world as well as in Pakistani population among patients of Dravet Syndrome, SCN1A gene was sequenced in one affected individual (proband) of each family. However, after review of literature for SCN1A exons i.e. 05, 15, 21, 25 and 26 were selected for initial screening to rule out the involvement of frequently found SCN1A mutations.

Sanger sequencing of the probands of each family resulted in the identification of two homozygous variants in family B i.e. g.82579A>G and g.19189-19190insG, one insertion g.64092_64093isnA and one homozygous variant g.82579A>G in family D. All these variants lie in the intronic or UTR regions of the SCN1A gene.

The variant g.82579A>G was identified in two families. i.e., family B and D. This variant was not found on gnomAD. In silico analysis tools like Mutation taster, support

that variant g.82579A>G is disease causing variant and affects splicing by altering splice donor or acceptor site. However, the CADD score of this variant is 14.7 which is on borderline to call a variant deleterious. Splice AI and pangolin predicted this variant can alter splice donor site, which is almost 57 bp away, but the score is low i.e., 0.02. Franklin predicted this variant is non-coding or synonymous, is not located in a splice region and not predicted as splice-altering consequence.

Another non coding variant (g.82577G>A), two base pairs away, is already reported in this region with rsID number rs948256829. The physical location of g.82577G>A is chr2: 166847569C>T (hg19) and lies in 3'UTR of exon 26 of SCN1A gene. This variant is classified as variant of uncertain significance and submitted by Illumina Laboratory Services, Illumina on ClinVar. The variant was identified in an individual with Generalized epilepsy with febrile seizures plus, type 2 (GEFSP2). Using variant allele frequency, penetrance estimates, disease prevalence, and inheritance mode, a computed score was generated to determine whether variant (g.82577G>A) frequency is high to be causative of the disease. Considering the score, this variant cannot be ruled out as potential cause of disease, hence further investigation into its association with disease is warranted. This variant is also classified as a variant of uncertain significance by ACMG.

ACMG also classifies the variant identified in this study i.e., g.82579A>G, as variant of uncertain significance. Based on these results it is considered of uncertain significance, which needs to be validated by further studies.

The variant g.19189-19190insG is predicted as polymorphism by Mutation taster, and UMD predictor. Splice AI and pangolin predicted this variant can alter splice acceptor site, which is almost 315 bp away, but the score is low i.e., 0.01. Hence, it is not significant. According to these results and ACMG classification criteria it is considered benign.

The variant g.64092_64093isnA is predicted as polymorphism by Mutation taster, and UMD predictor. Splice AI and pangolin predicted this variant can alter splice acceptor site, which is almost 18 bp away, but the score is low i.e., 0.02 and 0.03, respectively.

Hence, it is not significant. According to ACMG and AMP classification it is also predicted as benign.

Despite prediction as disease causing by mutation taster and UMD predictor, the variant g.82579A>G can't be classified as pathogenic as no significant role in splicing is predicted by other tools used in this study to validate effect of this variant on splicing. So, additional studies are required. Whole exome sequencing or whole genome sequencing can be performed to confirm whether this Variant in SCN1A is responsible for causing epilepsy or other gene is involved.

In conclusion, by investigation of these five families we were unable to find any strong disease-causing variant. Other exons of SCN1A can be sequenced to identify the pathogenic variants in these families. Panel sequencing can also be performed of all the genes reported to cause epilepsy particularly genes involved in Dravet Syndrome.

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Haplopainter

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In-Silico PCR

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Mutation Taster

https://www.mutationtaster.org/

Oligo Calc

OligoCalc: Oligonucleotide Properties Calculator (northwestern.edu)

Primer 3

https://primer3.ut.ee/

Varsome

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CADD

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Franklin

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Analysis - UMD-Predictor Pro (genomnis.com)

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