Genetic Heterogeneity in Epilepsy Families of Pakistan

By

Kaenat khan

Reg. No. 628-FOS/MSBT/F2023

Supervisor
Dr. Shaheen Shahzad
Assistant Professor

Co- Supervisor
Dr. Asma Gul
Professor

Department of Biological Sciences

Faculty of Sciences

International Islamic University Islamabad

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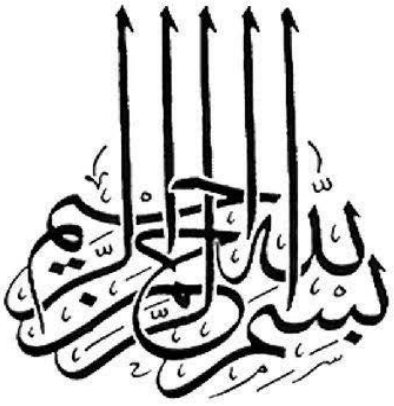
بسم الله الرحمن الرحيم

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DEDICATION

With deep gratitude and utmost respect, honor my parents and teachers with this piece, who have been my guiding lights throughout this journey. I dedicate this work with heartfelt gratitude and deep appreciation.

DECLARATION

I hereby declare that the work done on this MS Biotechnology research thesis "**Genetic Heterogeneity in Epilepsy Families of Pakistan**" is purely conducted by me. All the material is prepared by me and has not been copied from anywhere; however, some text and figures have been used which are properly referenced.

Kaenat khan

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LIST OF ABBREVIATIONS

Abbreviations	**Definition**
AEDs	Antiepileptic drugs
CECP	Comprehensive Epilepsy Control Programme of Pakistan
ADLTE	Autosomal dominant lateral temporal lobe epilepsy
GEFS	Genetic epilepsy with febrile seizures
JME	Juvenile myoclonic epilepsy
CAE	Childhood absence epilepsy
TLE	Temporal lobe epilepsy
IGE	idiopathic generalized epilepsy
BFNE	Benign familial neonatal epilepsy
NGS	Targeted Next-Generation Sequencing
GRL	Genomic Research Laboratory
gDNA	Genomic deoxyribonucleic acid
PCR	polymerase chain reaction
CDKL5	Cyclin-dependent kinase-like 5
SCN1A	sodium voltage-gated channel alpha subunit 1
GABRA1	gamma-aminobutyric acid A receptor subunit alpha
KCNQ2	potassium voltage-gated channel subfamily Q member 2
LGI1	Leucine-Rich Glioma-Inactivated 1
JME	juvenile myoclonic epilepsy
WES	Whole-Exome Sequencing

ASD	Autism spectrum disorders
ID	Intellectual disability
DD	Developmental delay
SPTBN5	Spectrin Beta, Non-Erythrocytic 5
CNTNAP2	Contactin-Associated Protein-Like 2
GWAS	Genome-wide association studies
mm	Millimeter
µm	Micrometer
TE	Tris EDTA
SDS	Sodium dodecyl sulfate
MgCl2	Magnesium chloride
EDTA	Ethylene diamine tetra acetic acid
NACL	Sodium chloride
1XTBE	Tris Boric EDTA

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ABSTRACT

Epilepsy is a prevalent neurological disorder that impacts around 50 million individuals worldwide. with a particularly high prevalence in countries with low and middle incomes, such as Pakistan. In Pakistan, the condition impacts an estimated 9.99 individuals per 1,000, which is significantly higher than the global average. A considerable portion of these cases has a familial linkage, often associated with inherited genetic factors, which is exacerbated by the country's high incidence of consanguineous marriages. The present research focuses to investigate the genetic heterogeneity in familial epilepsy from Pakistan by discovering new variant of the known candidate gene through Sanger sequencing technology. The study will include selecting families with a recorded history of epilepsy, conducting clinical assessments, extracting DNA, then PCR was performed and selected genes were checked for mutations through Sanger sequencing.

Two Pakistani families with known history of epilepsy were sampled from Islamabad, the three exons of SCN1A gene, i.e., 5, 21, and 26 were selected as candidate and subjected to sanger sequencing. After the careful analysis of Sanger sequencing result no pathogenic variants in the targeted regions were identified. These results suggest that the selected exon of SCN1A gene do not contribute to epilepsy in these families, thus emphasizing the idea of considerable genetic diversity in epilepsy. This indicates that mutations in these regions are unlikely to account for the epilepsy seen in families EP1 and EP2. The absence of variants in these exons indicates that the mutations might possibly be in unscreened exons, intronic regions, regulatory elements, or possibly other genes. Further exploration via whole-exome sequencing (WES) or whole-genome sequencing (WGS) is required to discover the genetic factor contributing to the epileptic traits in these families

1.Introduction

Epilepsy is a diverse group of neurological condition characterized by recurrent, unprovoked seizures cause from abnormal electrical activity within the brain. It is a common chronic illness that can affect peoples of any age regardless of gender. (Cascino, 1994). The Worldwide Impact of Epilepsy Report indicates that around 13 million people are newly diagnosed with epilepsy each year. Worldwide, over 50 million peoples currently live with epilepsy, and around 125,000 people die each year due to problems associated to epilepsy. Significantly, about 80% of these cases occur in low- and middle-income nations (Singh *et al.,* 2020). Although 20–30% of epilepsy cases are due to acquired conditions like stroke, tumors, or head injuries, one or more genetic factors are thought to be responsible for the remaining 70–80%. (Hildebrand *et al.,* 2013).

Epilepsy is a significant issue for public health in Pakistan, not only because of the physical problems it causes but also because of the stigma and lack of available healthcare resources. A crucial yet frequently neglected aspect of epilepsy cases in Pakistan is genetic heterogeneity, which refers to the presence of different genetic mutations that can lead to similar epileptic symptoms within and among families. This variation in genetic causes is especially relevant in Pakistan, given the high prevalence of consanguineous marriages, which raises the chances of inheriting autosomal recessive disorders, including different types of epilepsy. Consanguinity, defined as marriages between closely related individuals, is common in various regions of Pakistan, especially in rural and tribal regions. This cultural practice raises the risk of inherited disorders like epilepsy by raising the possibility that both parents may have the same genetic mutation.Current genetic investigations employing methods such as whole-exome sequencing (WES) have revealed significant genetic variations among Pakistani families impacted by epilepsy. One study identified new homozygous mutations in genes including COL18A1, UFSP2, ZFYVE26, and ATP13A2 spread across four distinct families. These genetic changes were related not only to epilepsy but also to developmental and intellectual disabilities, along with speech delays suggesting that epilepsy frequently coexists with wider neurodevelopmental challenges (Yasin *et al.,* 2024).

Further evidence of genetic diversity comes from other research that determined new variants in genes such as CNTN2, CARS2, ARSA, and CLCN4 within consanguineous families showing epileptic symptoms. These mutations presented diverse clinical manifestations even among members of the same family. Some people experienced seizures in their early childhood, while others did so in their adolescence or early adulthood. Even when involving the same gene, this diversity in severity and onset age demonstrates the complexity of the genetic basis of epilepsy, which is impacted by a number of additional genetic and environmental factors (Abdulkareem *et al.,* 2023).

An important gene in the study of epilepsy in Pakistan is EFHC1, which is connected to juvenile myoclonic epilepsy (JME). In research that involved 66 JME patients, several variants, including R159W and V460A, were revealed within this gene. Even though these variants were heterozygous, they played a vital role in causing seizures. Notably, these mutations were different from those typically found in Western populations, highlighting the necessity for genetic research tailored to specific populations to enhance diagnosis and treatment (Saleem *et al.,* 2021).

Understanding this genetic complexity is essential for accurate diagnosis, treatment, and counseling. Since there are several mutations that might cause epilepsy, a standardized diagnostic method frequently fails. Relying solely on clinical assessments may miss the genetic underpinnings, resulting in misdiagnoses or insufficient treatment. The adoption of advanced genetic methods such as whole-exome sequencing (WES) can improve the precision of diagnoses by identifying mutations specific to individual patients. This open the way for personalized treatments, which could involve gene-targeted drug therapies or lifestyle changes designed to improve outcomes.

The wider consequences of this research are substantial. For example, couples especially those in consanguineous unions could gain from genetic counseling to better grasp the risk of transmitting epilepsy-related mutations. Raising public awareness and education campaigns could reduce the occurrences of genetic epilepsy by encouraging informed reproductive choices. Additionally, establishing national genetic databases and screening programs would facilitate early detection and preventive measures (Yasin *et al.,* 2024).

At present, Pakistan's healthcare system lacks the infrastructure required for extensive genetic testing and counseling. Nonetheless, identifying mutations associated with epilepsy lays the groundwork for

creating cost effective, population specific diagnostic tools. As more families undergo genetic screening, a clearer understanding of the genetic landscape of epilepsy in Pakistan will develop, ultimately enhancing patient care and informing public health initiatives (Riaz *et al.,* 2019).

1.1 Aim and Objectives

The main aim of the study is to investigate the genetic heterogeneity and identify novel gene variants responsible for familial epilepsy in Pakistani populations, contributing to the understanding of epilepsy genetics with following objectives.

- To identify families of epilepsy from selected region of Pakistan.
- To investigate the genetic heterogeneity of epilepsy among Pakistani families through comprehensive genetic and clinical analysis.
- To identify novel gene variants linked to familial epilepsy in Pakistan using Sanger Sequencing analysis.

2. Literature Review

Epilepsy is a long-lasting neurological condition characterized by repeated, untriggered seizures caused by irregular electrical signals in the cerebral cortex. A single seizure is not enough for a diagnosis; the condition is diagnosed when at least two unprovoked seizures occur (Fisher *et al.,* 2014). Approximately 50 million people are affected globally, with a higher prevalence in low- and middle-income nations (WHO, 2019). Research has identified a significant link between consanguineous marriages and an increased risk of epilepsy, particularly within Middle Eastern populations (Bener *et al.,* 2006). The evidence for genetic predisposition is reinforced by the tendency for familial clustering, especially in idiopathic generalized epilepsy types such as childhood absence epilepsy and juvenile myoclonic epilepsy (Helbig *et al.,* 2008). In communities where endogamy is prevalent, the chances of inheriting autosomal recessive mutations are markedly elevated due to shared ancestry, thereby raising the risk of developing epileptic conditions (Khan *et al.,* 2021).

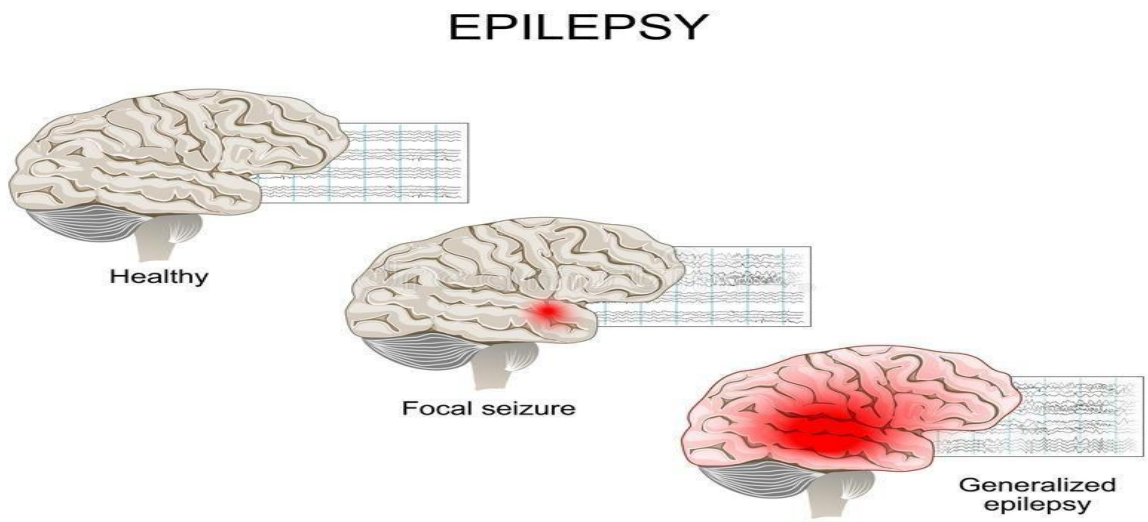

Figure 1:- Illustration of epilepsy progression, showing differences between a healthy brain, a focal seizure, and generalized epilepsy (Stafstrom & Carmant, 2015).

2.1 Classification of Epilepsy

Epilepsy is classified based on the nature of the seizures and their underlying causes. Medical professionals usually divide epilepsy into the following primary categories:

2.1.1 Focal (Partial) Epilepsy: Seizures in this type originate from a particular region in the brain. They can be further divided into:

- Focal onset aware seizures, where the individual remains completely conscious.
- Focal onset impaired awareness seizures, where there is an alteration or loss of consciousness.
- Focal to bilateral tonic-clonic seizures, where abnormal brain activity spreads to both sides of the brain, resulting in generalized convulsions and loss of consciousness (Fisher *et al.,* 2017).

2.1.2 Generalized Epilepsy: This type of seizure starts simultaneously across both sides of the brain. Common variations include:

- Tonic-clonic seizures (Grand Mal): marked by a stiffening of the body followed by rhythmic shaking.
- Absence seizures (Petit Mal): characterized by short lapses of awareness without convulsions, primarily seen in children.
- Myoclonic seizures: quick, involuntary muscle spasms.
- Atonic seizures: abrupt loss of muscle tone leading to falls.
- Tonic seizures: sudden rigidity of muscles, which can also result in falling (Berg *et al.,* 2010).

2.1.3 Unknown Onset Epilepsy: The initial onset of the seizure is either not witnessed or cannot be clearly defined. This includes seizures that are challenging to classify as either focal or generalized (Fisher *et al.,* 2017).

2.1.4 Idiopathic Epilepsy: This type does not have a known cause and is often diagnosed in the presence of a family history of epilepsy, typically beginning during childhood or adolescence (Berg *et al.,* 2010).

2.1.5 Symptomatic Epilepsy: Also referred to as secondary epilepsy, this form has a known origin such as traumatic brain injury, tumors, infections, or a stroke, and often presents with distinct and consistent seizure patterns (Hauser *et al.,* 1990).

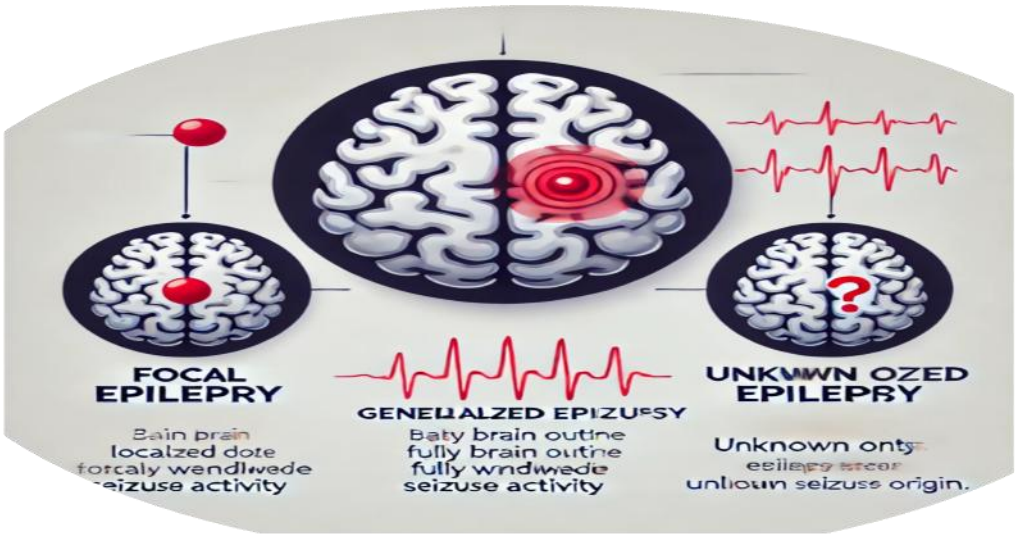

Figure 2:- Illustrating different types of epilepsy, with clear distinctions for focal, generalized, and unknown onset epilepsy (Fisher *et al.,* 2017).

2.2 Epilepsy in the Pakistani Population: Prevalence and Challenges

In Pakistan, epilepsy is a prevalent neurological disorder, affecting approximately 9.99 per 1,000 people, which decodes to an estimated 2 million people across the nation. A notably high prevalence is observed among those under the age of 30, indicating a heavier disease burden on the younger demographic (Khatria *et al.,* 2003).

2.2.1 Treatment Gap and Access to Care

Although antiepileptic drugs (AEDs) are available, a large part of the population remains untreated, mostly in rural areas. Studies show that about 98.1% of individuals with active epilepsy in rural locations do not receive proper treatment, in contrast to 72.5% in urban areas. Contributing elements include a lack of neurologists, limited medication supplies, insufficient

healthcare infrastructure, and inadequate access to specialized services (Mogal *et al.,* 2020).

2.2.2 Stigma and Misconceptions

The prevalent societal stigma and misconceptions about epilepsy make significant obstacles to effective management. Many individuals encounter discrimination and social elimination, which discourages them from seeking medical assistance. Research shows that the societal view of epilepsy as a taboo condition leads to its concealment, complicating public health initiatives (Mogal *et al.,* 2020).

2.2.3 Initiatives and Recommendations

Efforts to tackle these challenges have involved public awareness campaigns and national programs like the Comprehensive Epilepsy Control Programme of Pakistan (CECP). These initiatives have been aimed at reducing the treatment gap by increasing awareness, establishing specialized centers, and educating communities. Preliminary findings suggest enhanced acceptance and a decline in untreated cases (Mogal *et al*., 2020).

To further lessen the impact of epilepsy in Pakistan, a comprehensive strategy is crucial. This should involve strengthening the healthcare system, ensuring broad and affordable access to AEDs, training more healthcare providers focused on epilepsy care, and continuing public education efforts to counter myths and diminish stigma (Bashir *et al.,* 2020).

2.3 Genetics of Epilepsy

Genetic factors are important in the onset of many types of epilepsy, especially generalized epilepsies. The genetic framework of epilepsy can be generally divided into three classifications: single-gene (monogenic) variations, complex polygenic inheritance, and chromosomal abnormalities (Ottman *et al.,* 2010).

2.3.1 Monogenic (Single-Gene) Epilepsies

Certain uncommon epilepsy syndromes result from mutations in a specific gene, usually those that influence ion channel activity, synaptic signaling, or the regulation of neurotransmitters. Notable examples include:

2.3.1.1 SCN1A: Changes in this gene are frequently linked to Dravet syndrome and various other severe epileptic encephalopathies (Depienne *et al.,* 2009).

2.3.1.2 KCNQ2/KCNQ3: These genes are linked with benign familial neonatal epilepsy (BFNE) (Singh *et al.,* 1998).

2.3.1.3 LGI1: Variants cause autosomal dominant lateral temporal lobe epilepsy (ADLTE) (Kalachikov *et al.,* 2002).

2.3.1.4 GABRG2: Mutations in GABRG2 gene result in genetic epilepsy with febrile seizures plus (GEFS+) (Baulac *et al.,* 2001).

2.3.2 Polygenic and Complex Epilepsies

The most common epilepsy types, such as idiopathic generalized epilepsy (IGE) and focal epilepsies, mostly result from the interaction of multiple genetic variants along with environmental factors. These include:

- Juvenile Myoclonic Epilepsy (JME)
- Childhood Absence Epilepsy (CAE)
- Temporal Lobe Epilepsy (TLE)

Genome-wide association studies (GWAS) have uncovered numerous small effect risk alleles, supporting the theory that these forms are polygenic in nature (ILAE Consortium, 2018).

2.3.3 Chromosomal Abnormalities and Structural Variants

Structural alterations in chromosomes, such as microdeletions or duplications, can also lead individuals to epilepsy. Examples include:

2.3.3.1 microdeletion 15q13.3: Associated with generalized epilepsy and intellectual disability (Helbig *et al.,* 2009).

2.3.3.2 microdeletion1q21.1 : Linked to epilepsy and other neurodevelopmental disorders (Mefford *et al.,* 2008).

2.3.3.3 deletion 22q11.2: Found in syndromes that include epilepsy as part of the broader clinical picture (McDonald *et al.,* 2015).

2.4 Genes involved in epilepsy

The analysis of candidate genes is a frequently used approach in pinpointing genetic factors linked to epilepsy through both case-control and family-oriented studies. Due to the genetic diversity and complex nature of epilepsy, it has been challenging to identify definitive genetic risk factors. In such complicated conditions, the candidate gene strategy is advantageous as it allows scientists to investigate large populations for variations in genes that are believed to affect neuronal excitability, neurotransmission, and the functioning of ion channels (Guo *et al.,* 2017).

2.4.1 SCN1A (Sodium Voltage-Gated Channel Alpha Subunit 1)

The SCN1A gene is situated on human chromosome 2q24.3 and encodes the α-subunit NaV1.1, a crucial voltage-gated sodium channel for neuronal excitability (Escayg *et al.,* 2010). This gene consists of 29 exons (Claes *et al.,* 2001). Variants in SCN1A are among the most significant and extensively researched discoveries in epilepsy genetics. SCN1A is recognized as the leading candidate gene for monogenic epilepsies, notably Dravet syndrome (severe myoclonic epilepsy of infancy, SMEI) and other genetic epilepsy disorders (Mulley *et al.,* 2005). The NaV1.1 channel produced by SCN1A is mainly found in inhibitory GABAergic interneurons, particularly concentrated at the axon initial segment (AIS), where it plays a key role in the initiation and spreading of action potentials (Ogiwara *et al.,* 2007).

Loss-of-function mutations in SCN1A diminish sodium current in GABAergic interneurons, which results in a decreased inhibitory tone and increased neuronal hyperexcitability, making the individual more susceptible to seizures (Catterall, 2018).

Over 1,250 variants of SCN1A have been documented, which include missense, nonsense, frameshift, and splice-site mutations (Parihar & Ganesh, 2013). Although early research indicated that SCN1A mutations were infrequent in generalized epilepsies (Escayg *et al.,* 2001), later studies have validated

their significant role in Dravet syndrome, generalized epilepsy with febrile seizures plus (GEFS+), and familial epilepsy syndromes across various populations (Marini *et al.,* 2011; Meng *et al.,* 2015).

2.4.2 SCN1B (Sodium Voltage-Gated Channel Beta Subunit 1) Gene

The SCN1B gene is located on chromosome 19q13.12, covering approximately 34 kb and consisting of 6 exons. It produces two protein variants, β1 and β1B subunits, which serve as accessory units for sodium channels (Wallace *et al.,* 1998). These β-subunits modulate the activity of sodium channel α-subunits (such as SCN1A, SCN2A, and SCN5A) by influencing their opening and closing mechanisms, thereby impacting neuronal excitability (Brackenbury *et al.,* 2011). They also function as cell adhesion molecules, assisting in axon guidance and the organization of neuronal networks (Patino *et al.,* 2010). Alterations in SCN1B are associated with epilepsy disorders, including Generalized Epilepsy with Febrile Seizures Plus (GEFS+) and Dravet syndrome (Wallace *et al.,* 1998; Patino *et al.,* 2009). These alterations may disrupt sodium channel function or inhibit cell adhesion, resulting in heightened neuronal excitability (Malley, 2015). A notable mutation, C121W, hinders the normal functioning of the β1-subunit and is linked to febrile seizures and epilepsy (Wallace *et al.,* 1998). Additional variants such as p.R85C and p.R85H have been identified in cases of severe epileptic encephalopathies (Scheffer *et al.,* 2007).

2.4.3 SCN2A (Sodium Voltage-Gated Channel Alpha Subunit 2) Gene

The SCN2A gene, found on chromosome 2q24.3, encodes the NaV1.2 protein, a key component of the voltage-gated sodium channel. This gene extends over 300 kb of DNA and comprises 27 exons (Liao *et al.,* 2010). NaV1.2 is predominantly expressed in the brain's excitatory neurons, particularly at the axon initial segment (AIS) and nodes of Ranvier, where it plays a crucial role in initiating and conducting action potentials (Shi *et al.,* 2012). Mutations in SCN2A are closely associated with various forms of epilepsy, ranging from mild neonatal seizures (BFNIS) to severe early infantile epileptic encephalopathy (EIEE) (Liao *et al.,* 2010; Howell *et al.,* 2015).

These mutations can result in either: Gain-of-function: Sodium channels become hyperactive, heightening neuronal excitability and contributing to early-onset epilepsy. Loss-of-function: Sodium channel function is diminished, often associated with autism spectrum disorder (ASD) and intellectual disability (Shalom *et al.,* 2017). Some well-characterized mutations, including p.R1882Q and

p.R853Q, enhance persistent sodium currents, causing excessive neuronal firing and precipitating seizures (Howell *et al.,* 2015; Wolff *et al.,* 2017).

2.4.4 KCNA1 (Potassium Voltage-Gated Channel Subfamily A Member 1) Gene

The KCNA1 gene encodes the Kv1.1 α-subunit of a voltage-gated potassium channel, situated on chromosome 12p13.32. It extends over approximately 24 kb of DNA and is composed of 2 exons (Browne *et al.,* 1994). Kv1.1 channels are extensively found in the brain and peripheral nerves, particularly in axons and presynaptic terminals. They play a crucial role in regulating neuronal excitability by managing action potential repolarization and neurotransmitter release (Imbrici *et al.,* 2006). Mutations in KCNA1 lead to various neurological disorders, notably episodic ataxia type 1 (EA1), characterized by recurrent episodes of ataxia and persistent muscle twitching (myokymia) (Browne *et al.,* 1994). Variants in this gene have also been associated with epilepsy, as dysfunctional Kv1.1 can prolong neuronal depolarization, thereby raising the risk of seizures (Zuberi *et al.,* 1999). Pathogenic variants such as R417X and V408A interfere with channel gating or surface expression, resulting in neuronal hyperexcitability (Eunson *et al.,* 2000). Certain mutations diminish potassium current, delaying repolarization and encouraging excessive firing, while others modify gating kinetics, disrupting inhibitory control (Imbrici *et al.,* 2006).

2.4.5 KCNA2 (Potassium Voltage-Gated Channel Subfamily A Member 2) Gene

The KCNA2 gene is responsible for encoding the Kv1.2 α-subunit of voltage-gated potassium channels and is situated on chromosome 1p13.3. It spans more than 20 kb of genomic DNA and consists of two exons (Browne *et al.,* 1994). Kv1.2 channels are predominantly found in the hippocampus and cerebellum, where they play a crucial role in regulating neuronal excitability, repolarization, and the shaping of action potentials (Imbrici *et al.,* 2016). Mutations in KCNA2 are linked to developmental and epileptic encephalopathy (DEE) as well as early-onset epilepsy, which is frequently associated with ataxia, motor difficulties, and cognitive challenges (Syrbe *et al.,* 2015). Both gain-of-function and loss-of-function mutations can occur: GoF mutations enhance potassium currents, leading to neuronal hypoexcitability and ataxia. Conversely, LoF mutations reduce potassium currents, resulting in delayed repolarization and seizures (Imbrici *et al*., 2016). A prominent variant is p.Arg297Gln (R297Q), which is associated with severe early-onset encephalopathy (Syrbe *et al.,*

2015). Another variant, p.Pro405Leu (P405L), also impairs channel function and leads to epilepsy with ataxia (Masnada *et al.,* 2017).

2.4.6 GABRA1 (Gamma-Aminobutyric Acid Type A Receptor Subunit Alpha1) Gene

The GABRA1 gene encodes the α1 subunit of the GABAA_AA receptor, situated on chromosome 5q34–q35. This gene covers around 25 kb of genomic DNA and includes 9 exons (Macdonald *et al.,* 2010). GABAA_AA receptors function as ligand-gated chloride ion channels, facilitating most rapid inhibitory neurotransmission within the central nervous system. The α1 subunit is one of the most prevalent isoforms, found extensively in the cerebral cortex, hippocampus, thalamus, and cerebellum, where it is essential for synaptic inhibition, the regulation of neuronal excitability, and the maintenance of the excitatory inhibitory balance (Olsen & Sieghart, 2009). Pathogenic mutations in GABRA1 have been closely linked to epileptic encephalopathies and genetic generalized epilepsies, such as childhood absence epilepsy, juvenile myoclonic epilepsy, and Dravet-like syndromes (Cossette *et al.,* 2002; Hirose, 2014). Most variants that cause disease are missense mutations that change channel gating, decrease surface expression, or hinder GABA binding, ultimately leading to reduced inhibitory signaling and an increased risk of neuronal hyperexcitability (Macdonald *et al.,* 2010).

2.4.7 GRIN2A (Glutamate Ionotropic Receptor NMDA Type Subunit 2A) Gene

The GRIN2A gene, found on chromosome 16p13.2, encodes the GluN2A subunit of the NMDA receptor, which is essential for communication within the brain, as well as learning, memory, and synaptic development (Endele *et al.,* 2010; Paoletti *et al.,* 2013). Variations in the GRIN2A gene are closely associated with the epilepsy-aphasia spectrum (EAS), which encompasses disorders like Landau-Kleffner syndrome (LKS), continuous spike-and-wave during sleep (CSWS), and Rolandic epilepsy with speech difficulties (Lesca *et al.,* 2013). Mutations that result in loss of function diminish receptor activity, leading to impaired brain signaling and language difficulties. In contrast, gain-of-function mutations enhance receptor activity and calcium influx, resulting in increased brain activity and seizures (Endele *et al.,* 2010; Lesca *et al.,* 2013). Notable variants include N615K and R518H, which are associated with severe forms of epileptic encephalopathy (Lemke *et al.,* 2014). The presence of diverse mutations in GRIN2A can produce varying effects, which accounts for the broad spectrum of symptoms observed in patients (Platzer *et al.,* 2017). While not as common as mutations in sodium or potassium channels, GRIN2A remains one of the significant glutamatergic genes linked to epilepsy,

particularly those that impact speech and language (Carvill *et al.,* 2013; Lesca *et al.,* 2013).

2.5 Genetic Heterogeneity in Epilepsy

Recent studies, especially regarding South Asian populations, have brought to light the genetic variability that underlies epilepsy. In families from Pakistan, mutations in genes for example CNTNAP2 and SPTBN5 have been discovered. These genes are crucial for brain development and neuronal function (Yasin *et al.,* 2024).

CNTNAP2: Crucial for neuronal connectivity. Variations have been linked with epilepsy, autism spectrum conditions, intellectual challenges, and speech disorders.

SPTBN5: Essential for preserving cytoskeletal stability in neurons. changes in this gene are linked to seizures and broader neurological impairments.

The effects of these variations vary among individuals, leading to different clinical consequences, ranging from mild epilepsy to severe developmental conditions.

2.5.1 Role of Consanguinity in Genetic Heterogeneity

In Pakistan, the practice of marrying close relatives especially first and second cousins is common. While this practice toughens family bonds, it also increases the likelihood of offspring inheriting homozygous harmful mutations, which increases the risk of inherited disorders like epilepsy (Modell *et al.,* 2002).

2.5.1.1 Impact on Genetic Disorders

The increased genetic relatedness in blood-related unions raises the probability of offspring inheriting identical copies of recessive alleles, leading to a higher occurrence of genetic disorders (Tadmouri *et al.,* 2009).

In the context of epilepsy, studies have proved that consanguinity contributes to the genetic heterogeneity observed in epilepsy cases within Pakistani families. For instance, a study involving consanguineous Pashtun families identified novel genetic variants associated with epilepsy, highlighting the role of consanguinity in revealing rare genetic mutations (Younus *et al.,* 2020).

2.5.1.2 Genetic Counseling and Public Health Initiatives

To lessen the genetic risks related with consanguinity, genetic counseling plays an important role. Counseling services before marriage and conception, especially for individuals with a family history of epilepsy, can help in informed decision making. Public health campaigns promote awareness of the hereditary risks and encourage voluntary genetic screening are crucial steps toward prevention and primary intervention (Hamamy, 2012).

2.6 Significance of Studying Genetic Variability in Pakistani Epilepsy Patients

Epilepsy is a complex neurological condition with a significant genetic component. Investigating genetic variability among Pakistani epilepsy patients is important due to several key causes.

2.6.1 Identification of Novel Genetic Variants

Reviewing genetic diversity in epilepsy patients can help identify previously unidentified genetic variants linked with the disorder. Pakistan has a unique genetic landscape due to its diverse cultural groups and high consanguinity rates. This rise the likelihood of discovering rare or population specific mutations that contribute to epilepsy. Such discoveries can enhance the global understanding of the genetic architecture of epilepsy, paving the way for more precise diagnostic methods (Helbig *et al,.* 2020).

2.6.2 Development of Targeted Therapies

Understanding the genetic basis of epilepsy allows the development of personalized treatment policies. Certain genetic mutations influence drug response, making some anti-epileptic drugs (AEDs) more effective or ineffective for specific individuals. By identifying these mutations, clinicians can tailor treatments to improve efficacy, minimize adverse effects, and enhance patient outcomes (Lindy *et al,.* 2018). This approach aligns with precision medicine, which aims is to improve healthcare based on an individual's genetic profile.

2.6.3 Public Health Implications and Genetic Counseling

In Pakistan, where consanguineous marriages are common, there is an enlarged risk of inheriting recessive genetic disorders, including epilepsy. Genetic studies can help identify at risk families and facilitate early interventions through genetic counseling. This can aid in reducing the incidence of

epilepsy in future generations by informing reproductive choices and promoting awareness about genetic risks (Nasir *et al,.* 2021). Moreover, such research can guide public health policies by integrating genetic screening programs to manage epilepsy more effectively at a national level.

2.7 Methodological Approaches in Genetic Research on Epilepsy

The practice of advanced sequencing technologies has revolutionized epilepsy research, specifically in countries with high consanguinity like Pakistan. The main methods include Whole-Exome Sequencing (WES), Targeted Next-Generation Sequencing (NGS), and Sanger Sequencing.

2.7.1 Whole-Exome Sequencing (WES)

Whole-Exome Sequencing (WES) targets the genome's protein coding regions known as exons which make up just 1–2% of the genome but account for roughly 85% of mutations accountable for human diseases (Bamshad *et al.,* 2011). In the field of epilepsy research, WES has been contributory in discovering both established and newly associated genes, mainly in populations with high rates of consanguinity, where homozygous disease-causing variants are more common (Rehman *et al.,* 2015).

Amongst Pakistani families, WES has recognized rare single-gene causes of epilepsy, including mutations in **SLC2A1**, **PNKP**, and **ASNS**, leading to higher diagnostic precision and enabling more personalized treatment methods (Nasir et al., 2021). The technique has confirmed that it is especially valuable in detecting autosomal recessive mutations that are more frequently observed in communities practicing endogamous marriages (Rehman *et al.,* 2015).

2.7.2 Targeted Next-Generation Sequencing (NGS)

Targeted Next-Generation Sequencing (NGS) emphases on analyzing a specific set of genes that are already known to be associated with epilepsy. This approach is especially useful in genetically heterogeneous populations like Pakistan, where numerous rare variants may contribute to the clinical presentation of the disease (Lindy *et al.,* 2018). Compared to whole-exome sequencing (WES) or whole-genome sequencing (WGS), targeted NGS is a more cost-effective and time-saving technique, yet it still provides high accuracy in detecting pathogenic variants in established epilepsy-related genes such as SCN1A, KCNQ2, CDKL5, and GABRA1 (Truty *et al.,* 2019).

Research conducted in Pakistan has confirmed that targeted NGS effectively uncovers mutations in patients with epileptic encephalopathies, highlighting its significance in both diagnostic and investigative settings (Nasir *et al.,* 2021).

2.7.3 Sanger Sequencing

Despite the widespread adoption of high-throughput techniques like Whole-Exome Sequencing (WES) and Next-Generation Sequencing (NGS), Sanger sequencing continues to play important role, especially in verifying potential disease-causing variants discovered through modern sequencing technologies (Sanger *et al.,* 1977). Due to its precision and dependability, it remains a standard technique for confirming mutations in both clinical and research applications.

In epilepsy research, Sanger sequencing is commonly used to:

- Verify variants of uncertain significance (VUS)
- Establish familial co-segregation of suspected mutations
- Identify common hotspot mutations in epilepsy-associated genes such as SCN1A and KCNQ2, particularly when targeted NGS is not accessible (Lindy *et al.,* 2018).

2.8 Significance of these Approaches

2.8.1 Early and Accurate Diagnosis*:* Advanced sequencing techniques significantly increase the ability to diagnose epilepsy precisely at an early stage, minimizing the chances of misdiagnosis and sidestepping ineffective or unnecessary treatments (Lindy *et al.,* 2018).

2.8.2 Personalized Treatment*:* Detecting the exact genetic alterations enables more personalized prescription of anti-epileptic drugs (AEDs), improving treatment effectiveness and patient outcomes (Helbig *et al.,* 2016).

2.8.3 ***Understanding Genetic Diversity****:* In Pakistani populations, these sequencing tools reveal unique, population-specific genetic variants connected to epilepsy that might be ignored in broader international studies (Nasir *et al.,* 2021).

2.9 Gap in Epilepsy Genetics Research in Pakistan

In Pakistan epilepsy is a significant neurological disorder and its underlying causes depends heavily on genetic contributions. In-depth genetic studies of epilepsy are still scarce in the nation, despite the fact that the disorder is common and consanguineous marriages have a major influence on the genetic landscape (Khan *et al.,* 2022). Bridging this research gap is essential for enhancing clinical care and promoting the development of precision-based treatment approaches for epilepsy.

2.9.1 Factors Contributing to the Research Gap

2.9.1.1 Limited Resources and Funding Constraints

Large-scale genetic research has been hindered in Pakistan by a lack of funding, especially in epilepsy. While wealthy countries have made great strides by investing heavily in genome-wide association studies (GWAS) and cutting-edge sequencing technologies, Pakistan still faces difficulties because of a lack of funds for research and insufficient infrastructure.(Khan et al., 2020).

2.9.1.2 Lack of Specialized Research Facilities and Expertise

Genetic research requires access to advanced labs, experienced researchers, and cutting-edge sequencing technology, all of which are still scarce in Pakistan. The discovery of novel genetic variants connected to epilepsy is severely hampered by the majority of healthcare and research organizations' lack of equipment for whole-exome sequencing (WES), next-generation sequencing (NGS), and even Sanger sequencing (Baig *et al.,* 2016).

2.9.1.3Absence of Genetic Testing in Routine Clinical Practice

Unlike in Western nations, where genetic testing is integrated into epilepsy diagnosis and treatment, Pakistan has restricted accessibility to genetic screening for epilepsy patients. This results in a reliance on symptom-based diagnoses, leading to potential misclassification of epilepsy types and suboptimal treatment (Sheikh *et al,.* 2019).

2.9.1.4 Cultural and Ethical Considerations

In Pakistan, consanguineous marriages are common, increasing the likelihood of autosomal recessive mutations linked to epilepsy. Despite this, genetic counseling services are largely unavailable, and public awareness regarding hereditary health risks remains low. Additionally, ethical challenges and

Societal stigma surrounding genetic conditions often prevent families from engaging in genetic research (Ahmad *et al.,* 2021).

3. Materials and Methods

The current research was conducted in the Genomic Research Laboratory at the department of Biological Sciences international Islamic university Islamabad.This chapter details the materials and methods used to carry out the experiments.

The methodology used to include the experimental planning for the entire research has been discussed in detail. Table 3.1 and 3.2 illustrates the list of chemicals and list of apparatus used in the study.

The list of chemicals used during the study is shown in Table 3.1.

Sucrose	Tris-HCL
$MgCL_2$	Triton X-100
NaCl	EDTA
Phenol	Ethanol (Oxoid)
Chloroform	Isoamly alcohol
SDS	Acetic acid
Sodium acetate	Distilled water
Boric Acid	Spirit
Tris Base	Sodium EDTA
Agarose	Ethidium Bromide
Proteinase K	Isopropanol

The list of apparatus used during the study is shown in Table 3.2.

Flasks (100ml, 250ml, 500ml)	Incubator
Reagent Bottle (250ml)	Centrifuge machine
Falcon tubes (15, 50 ml)	Magnetic stirrer
Eppendorf	Spatula
Measuring cylinder	Pipette
Microwave oven	Weighing balance
gel electrophoresis apparatus	Drying oven
Refrigerator	EDTA tubes
Cotton	Aluminium foil
Blue tips	Yellow Tips

The Research work was approved by the Ethical Review Committee at International Islamic University, Islamabad. The workflow of the research is illustrated in figure 3.1.

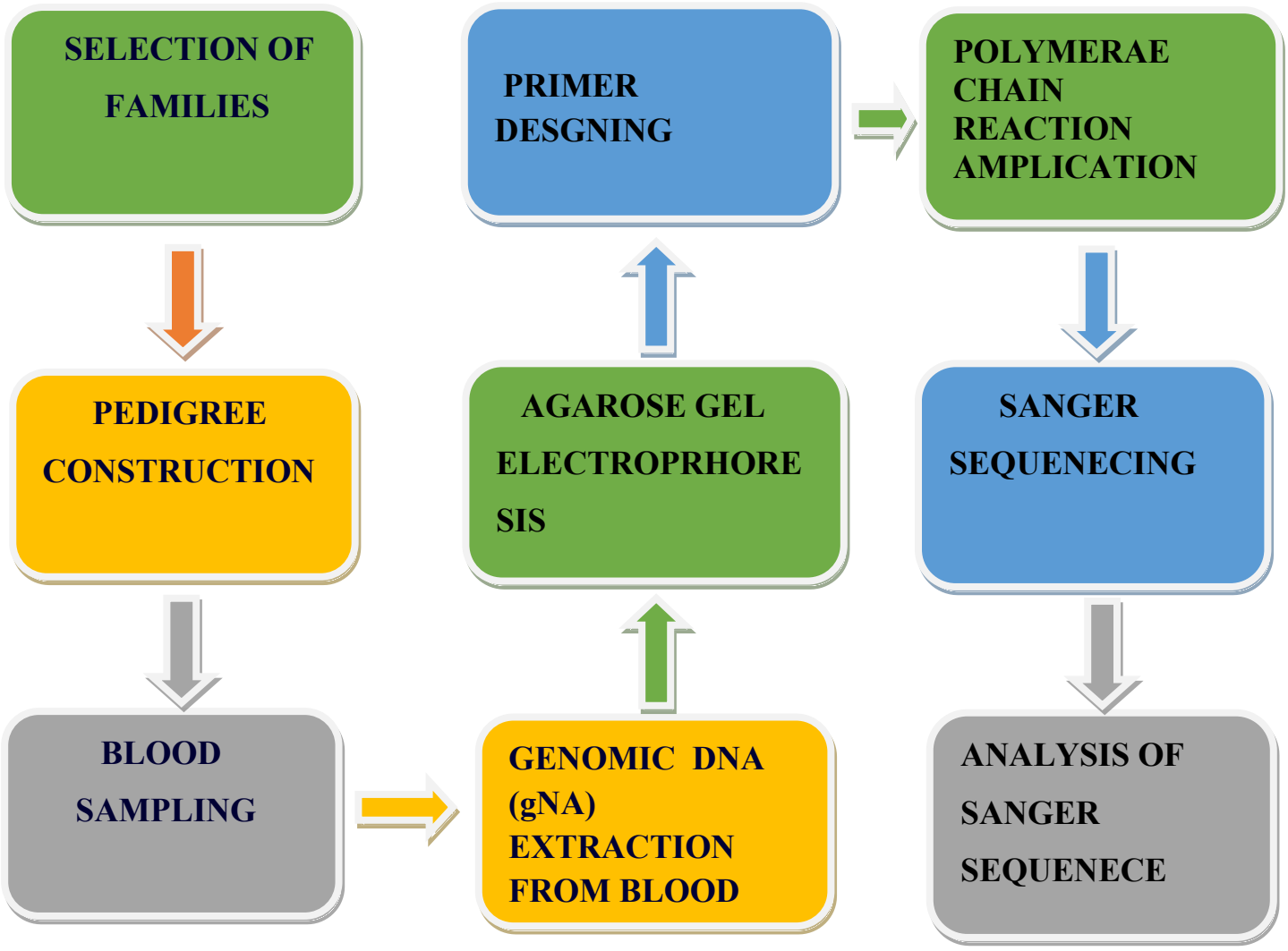

Figure 3.1:- Workflow showing the different steps carried out in the study

3.1 Selection of Families

Two Pakistani families, with multiple members affected with epilepsy, were selected from different region of Pakistan. All the family members taking part in the research project provided written informed consent for genetic/molecular analysis.

3.2 Data collection and Pedigree Construction

Data including both anthropometric and clinical as well as relevant details about both families were gathered through visits to their homes through properly designed questionnaire. Details such as the number of individuals impacted and their family medical history were collected. The parents provided their consent and had no issues regarding the collection of blood samples and the publication of photographs of the individuals affected.

Pedigrees were constructed using the method outlined by Bennett *et al.,* (2008) based on the information provided by knowledgeable elder members of all the families included in the study.

In the pedigree, squares represent males and circles represent females. Unfilled shapes are used for normal individuals and filled shapes for affected individuals. Crossed diagonal symbols are used to identify deceased people. Arabic numbers are used to identify people within a generation. Roman numerals represent different generations. Marriage is represented by the horizontal line, consanguinity by the double marriage line, and offspring by the vertical line. The line traced horizontally from the line of descent was known as the sibship line.

Clinical information, thorough medical histories were gathered from all the affected members of the study's participating families at local government hospitals and private clinics.

3.3 Blood Sampling

Blood samples were collected from all available individuals, both affected and unaffected, using 10 ml sterilized syringes via a venous approach. The collected blood samples were then placed into vacutainers containing ethylenediaminetetraacetic acid. (BD Vacutainer® K3 EDTA, Franklin Lakes NJ, USA) (Puri et al., 2009).and stored at 4°C at the Genomics Research Lab (GRL), Department of Biological Sciences, International Islamic University, Islamabad. Later, DNA was extracted from these blood samples.

3.4 Genomic DNA (gNA) Extraction from Blood

Phenol-chloroform method was used to extract DNA from the sample blood. This is considered the standard protocol for extraction of genomic DNA extraction (Sambrook *et al.,*1989).

3.4.1 DNA Extraction using Phenol-Chloroform Method

DAY 1:

- Blood samples were stored in EDTA tubes at 4°C for DNA extraction so they could acclimatize to room temperature for 60 minutes.
- From the vacutainer tube, 0.75 ml of blood was taken in an Eppendorf tube and 0.75 ml of solution A
- (0.32 M Sucrose, 10 mM Tris pH 7.5, 5 mM MgCl, and 1% (v/v) Triton X-100) was added to it and mixed.
- The tube was centrifuged at 13,000 rpm for 60 seconds. A dense pellet was obtained after discarding the supernatant.
- The pellet was resuspended in 400 ul of solution A and was centrifuged at 13,000 rpm for 60 seconds again. Supernatant was again discarded to obtain the nuclear pellet.
- This pellet was resuspended in 500 ul of solution B (10 mM Tris pH 7.5, 400 mM NaCI, 2 mM EDTA pH 8.0). 28 ul of 10% sodium dodecyl sulphate (SDS), and 15-20 ul of proteinase K were also added to the Eppendorf tube.
- The tube was then incubated at 37°C for 24 hours.

DAY 2:

- After incubation, 0.5mL (500 L) of a fresh mixture of solution C (Phenol) and solution D [Chloroform: Isoamyl alcohol (24:1)] of equal volume was added to the pellet and thoroughly mixed, 10 minutes centrifugation was done at maximum speed (13,000rm).
- After that, two clear (transparent), conspicuous layers were scrutinized in the micro-centrifuge tube. The upper layer (aqueous phase) containing genomic DNA was moved to a new sterilized Eppendorf tube.
- Then 0.5mL (500 uL) of solution D was added. Another 10 minutes centrifugation was performed, and the upper layer was collected in a new Eppendorf tube.
- Later, 55 L of sodium acetate (3M) with pH6 and 0.5ml (500 L) chilled isopropanol was added. The tube was inverted several times for DNA precipitation and was centrifuged for 10 minutes

at maximum speed (13,000rpm).

- 70% chilled ethanol (350) was added to the pellet, and centrifugation was done for 10 minutes at 13,000rpm.
- After removing the ethanol, the pellet was dried at 45°C for 10 minutes in drying oven . After DNA precipitation, it was dissolved in 20-200L of Tris-EDTA (TE) buffer.

The chemical compositions of the solutions and buffers used for genomic DNA extraction are provided in Table 3.3.

Solution A	Sucrose 0.32 M	It provides osmotic shocks that help in bursting of blood cells.
	10mM Tris (pH 7.5)	It increases membrane permeability of cell membrane
	1% (V/V Triton X-100)	It is used in lysis to extract to remove organelles from living cells and maintain pH of solution.
	$MgCl_2$ 5mM	It protects the DNA from DNases enzymes.
Solution B	2 mM EDTA (pH 8.0)	It acts as chelating agent for capturing metal ions such as Ca2+, Mg2+.
	400mM NaCl	It stabilizes DNA and helps remove protein.

	10 mM Tris (pH 7.5)	It increases membrane permeability of cell membrane.
Solution C	Phenol	It is required for purification of nucleic acids.
Solution D	Chloroform (24 volumes)	Chloroform separates DNA from proteins, helping to clean DNA samples.
	Isoamyl alcohol (1 volume)	It binds to lipids and protein and reduce foaming occurring at time of DNA extraction.
Proteinase K	50µg/ml	It breaks down protein and help to clean the DNA.
10% SDS	3g in 50ml of water	SDS free the DNA from histones and other proteins by denaturing it.
3M sodium acetate	Na-acetate 12.304g in 50 ml of water (pH 6)	It helps to neutralize the solution and improve DNA precipitation.
70% Ethanol	Mrck	It help precipitate DNA during extraction, making it visible and isolating it from solution.
TE Buffer	10mm Tris-HCL Ph (8)	It is used to increase the permeability of cell membranes.
	0.1 Mm EDTA	Chelating agent.

3.5 DNA Concentration and Purity determination

A quantitative analysis of DNA using spectrophotometry was conducted with a NanoDrop spectrophotometer. The NanoDrop ND-1000 employs a unique mechanism that allows it to hold approximately 1 µl of sample without the necessity for conventional containers like cuvettes and capillaries. Absorbance readings were taken at 260 and 280 nm (A260 and A280, respectively). The absorbance ratio (OD260/OD280) serves as an indicator of DNA purity. A ratio value between 1.8 and 2.0 is considered to reflect good quality, purified DNA. A ratio below 1.8 suggests the presence of protein contamination, while a ratio exceeding 2.0 indicates possible RNA contamination.

3.6 DNA Integrity

The integrity of the genomic DNA was assessed by examining DNA samples on a 1% agarose gel. To prepare the 1% agarose gel, 0.5 g of agarose was dissolved in 50 ml of 1X TBE using a microwave oven for 1-2 minutes. To stain the DNA, 5 µl of ethidium bromide was mixed into the gel. The gel solution was then poured onto a gel casting tray fitted with a comb to create wells and allowed to solidify for 15–20 minutes at room temperature. Once solidified, the gel was transferred to an electrophoresis tank containing 1X TBE buffer, which acts as the running buffer (Thermo Scientific, USA). An equal volume (5 µL) of purified DNA and 5 µL of loading dye (blue-colored bromophenol blue) were mixed to facilitate visualization of DNA migration. The electrophoresis was conducted at 120 volts for a duration of 30 minutes, after which it was visualized using a UV transilluminator, and the results were documented with gel imaging.

The composition of solutions required for agarose gel electrophoresis is given in Table 3.4.

Solution	***Composition***	***Function***
Agarose	*Polymer of agarobiose*	*Agarose separates nucleic acid like DNA based on size, charge and other physical properties.*
Gel loading dye	*0.25% Bromophenol blue, pH 3.0-4.6*	*It is a staining dye and direct the DNA from negative to positive electrodes.*
	40% sucrose	*It gives weigh to DNA.*
Ethidium bromide	*0.5ug/ml final concentration*	*It binds to DNA and emit fluorescence under UV light* .
5X TBE buffer	*54g Tris base 27g Boric acid 4.63g EDTA pH 8.3*	*Optimized for use in nucleic acid application.*

3.7 Primer Designing

For present study,SCN1A gene was selected for variant analysis. For selected exons of the gene, Primer3 (http://bioinfo.ut.ee/primer3-0,4.0/) site was opted to get primer sequence. From both sides of the selected exon 100bp region was also taken. A primer designing site provides primer sequence with necessary feature as GC contents (40- 60%), desired length (18-22), annealing temperature (58-62°C) and whether to use these designed sequences as forward primer or reverse primer. Blast alignment tool (BLAST) (https://genome.uesc.edu/cgi-bin/hgBlat) which is available on UCSG genome browser (https://genome.uesc.edu/) was employed to check specificity/compatibility of desired primer sequence. Amplification of

exons was carried out by using these primer sequences illustrated in table 3.7.

3.8 Polymerase Chain Reaction (PCR) Amplification

In the present study, a 50ul reaction mixture was prepared in PCR tubes (200ul Axygen, USA) to amplify the exons of the selected gene. Amplification was carried out in Thermocycler. Table 5 indicates the reagent and their volume used in PCR. The reaction was carried out by adding the mentioned reagents into PCR tubes accurately and carefully. Final volume kept being 50ul. For proper mixing of all the reagents in PCR tubes, they were centrifuged (short spin) for 15 seconds at 4000 rpm and extended to a multigene optimal thermocycler (BIOER XP cycler) for amplification process temperature and time arrangements of thermocycler are given in table 3.5.

The reagents and their respective volumes used for PCR amplification are given in Table 3.5

Constituents	**Quantity µl= microliter**
Genomic DNA	3µl
Reverse primer	3µl
Forward primer	3µl
PCR water	16µl
Tag master mix	25µl
Total volume	**50µl**

3.8.1 Conditions for PCR

- Double stranded DNA was denatured at 96°C for 5 minutes in the first cycle. Initial denaturation was followed by 30 amplification cycles.
- Each amplification cycle had three sub cycles. Every cycle included denaturation of double stranded DNA for 1 min at 96° C, Annealing of primers to their complementary DNA sequence for 1 min at 50-60° C and Cyclic elongation of desired DNA sequence from each primer for 1 min at 72°C.
- Final extension or polymerization to complete the amplification process was carried out at 72ºC for 7 minutes (Frey *et al.,* 2008).

The thermal cycling conditions for PCR amplification are provided in Table 3.6.

Steps	Temperature (ºC)	Time period (Min)
Initial denaturation	96º	5
Denaturation	96º	1
Annealing	Specific to the primer	1
Extension	72º	1
Final Extension	72º	7

Min= minute

3.8.2 Visualization of PCR product

To evaluate the PCR result (amplified), a 2% agarose gel was prepared using the identical procedure as for the 1% agarose gel above, with the exception that the agarose constituent was twofold. 5ul PCR was added to wells following the gel's solidification. A 5ul of 50bp DNA ladder (Biron, Germany) was placed into the first well to check the amplicon size.

Electrophoresis was arranged with gel to load, and TBE buffer (I X) was poured in tank and required conditions like controlled electric supply (100-125volts, 400mA for 35-40min.) were set. Image of gel was visualized through Gel Documentation System (Protein Simple, USA).

The primer sequences designed for the selected exons of the SCN1A gene are listed in Table 3.7.

Gene	Exon	Primer Sequence	Product Length (bp)
SCN1A	**5F**	5’ AGGGCTACGTTTCATTTGTA 3’	473
	5R	3’ TCAACTACTGCAAAGCCTTC 5’	
	21F	5’ GCCTTAAATTCAACAGCAAT 3’	662
	21R	3’ GAGGCCTATTTCTCTTGCAT 5’	
	26F	5’ TACTGTTCGGCCAAATGTGC 3’	935
	26R	3’ ACTGAGTTGCATCGGGATCA 5’	

3.9 Sanger Sequencing

After purification, the purified PCR products were exposed to Sanger sequencing using BigDye* X Terminator ™' Cycle Sequencing Kit v3.1 (ABI, Applied Biosystems) with the forward and reverse primers in separate reactions. The kit contains dNTPs, double dNTPs, Taq polymerase and buffer (MgCI2). In dye-terminator sequencing, a fluorescent dye is used to label each of the four dideoxynucleotides chain terminators, each of each producing light at various wavelengths. The reaction mixture and sequencing conditions were used according to the manufacturer's directions.

Sequencing Reaction Program:

1. 96°C for 30secs (denaturation)
2. 50°C for I5secs (annealing)
3. 60°C for 4mins (elongation)
4. Steps 1-3 are repeated to complete 25 cycles After sequencing; amplified products were purified by using BIGDYE® X Terminator™ Purification Kit. DNA of available affected and healthy members of all families were sequenced to validate the likely pathogenic mutations.

3.10 Analysis of Sanger Sequencing Results

The chromatogram obtained after sequencing was examined using the Bio Edit sequence alignment program. Additionally, the reference sequences of the SCN1A gene are obtained from the genomic database ENSEMBL (https://asia.ensembl.org/index.html) and compared with the sequencing results to identify any differences between the two sequences.

4. RESULTS

4.1 Study Subjects

For variant analysis of selected gene, two families of epilepsy were collected from different region of Pakistan. The patients were evaluated according to certain clinical parameters. Information about patients such as age, gender, marital status, family history of disease, onset of disease and treatment were collected through properly designed questionnaire.

4.2 Pedigree and Family description

4.2.1 Family EP1

The family EP 1 of epilepsy belongs to Islamabad, the federal capital of Pakistan. Appropriate information was gathered from the affected family members about the disease.

A pedigree of four generations was constructed as illustrated in (figure 4.1). Pedigree investigation show autosomal recessive mode of inheritance. The pedigree was composed of 34 members of the family including two affected males and two affected female, 18 unaffected females and 16 unaffected males. Blood samples were obtained from 11 individuals. Family samples includes 3 affected individuals and eight normal individuals.

The symptoms of affected member of the family were investigated carefully (table 4.1). The clinical parameters were also taken, and a questionnaire was designed to gather information about epilepsy. This family was diagnosed with the help of neurologist. Electroencephalography test was used as the diagnostic marker test for the epilepsy. This test shows that affected person have any abnormalities in brain or not.

The affected members of the family included IV-1A, IV-4A, III-6Aas mentioned in pedigree.

4.2.2 Clinical observations

Affected individual IV-1A, diagnosed with generalized epilepsy before the age of 12. In start at age 2, she had frequent seizures, most of which happened while she was sleeping.

Both normal and abnormal EEG recordings were made at different ages. She also has a history of head trauma and cerebral palsy. The duration of seizures ranged from a few minutes to three hours, and they were followed by weakness and exhaustion. She was being treated with a variety of antiepileptic drugs, such as Vepridone, Tegretol, Rivotril, and Epival Syrup.

Other affected individual IV-4A was diagnosed with epilepsy at the age of 13. Her seizures were rare, and the nature of her epilepsy was not specified. Her seizures were triggered by stress, specific foods and beverages, and hormonal changes, while the EEG results were normal. She reported occasional dizziness as a side effect of the drug, Epival (250 mg) and Lerace (250 mg) once daily.

Affected individual III-6A was diagnosed with secondary epilepsy at the age of seventeen. He experienced infrequent seizures, which mostly happened when he neglected to take his medicine. The results of his EEG were normal. Symptoms include lack of sleep, drowsiness.

The clinical characteristics of the affected individuals from Family EP1 are summarized in Table 4.1.

Individual ID	IV-1A	IV-4A	III-6A
Age at Onset	2 years	13 years	17 years
Epilepsy Type	Generalized epilepsy	not mention	Secondary epilepsy
Seizure Frequency	High frequency	Rare	Rare
Triggers	Often during sleep	Stress, certain foods/drink	missed medication, lack of sleep
EEG Findings	Abnormal	Normal	Normal
Comorbidities	Cerebral palsy	-	-
Duration of Seizures	Few minutes to 3 hours	Not specified	Not specified
Current Medication & Dose	Epival Syrup, Tegretol, Rivotril, Vepridone	Epival 250 mg OD, Lerace 250 mg OD	Depekine 500 mg OD
Reported Side Effects	Fatigue, weakness	Occasional dizziness	Drowsiness

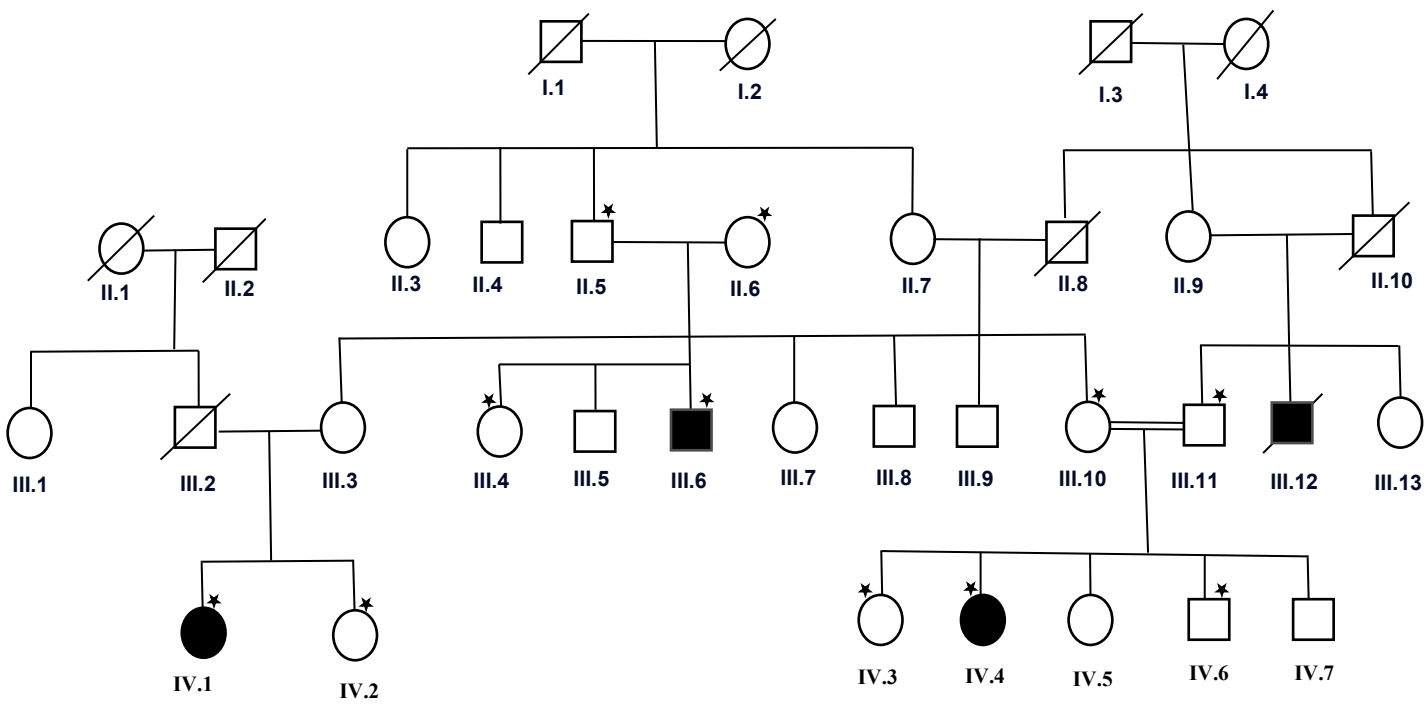

Figure 4.1:- pedigree of family EP 1 segregating Autosomal recessive Epilepsy. The samples were collected from the members that are marked with (*) in the pedigree

4.2.3 Family EP 2

The family EP2 of epilepsy belongs to Islamabad. The pedigree of the family was constructed by taking information from the family members through properly designed questionnaire. The pedigree is five generations as illustrated in (Figure 4.1). Pedigree investigation show autosomal recessive mode of inheritance. The pedigree comprised of 51 members including one affected male and two affected females, 21 normal females and 27 normal males. Blood samples were obtained from the five individuals of family marked with asterisk (*) in the pedigree diagram.

The symptoms of affected member of the family were investigated carefully (table 4.2). This family was diagnosed with the help of neurologist. Electroencephalography test was used as the diagnostic marker test for the epilepsy. This test shows that affected person have any abnormalities in brain or not.

The affected members of the family included Individual V-2 and Individual IV-9 in the pedigree.

4.2.4 Clinical Observation

Affected individual V-2A, was diagnosed with epilepsy at the age of 14 years. seizures at the age five years with brief episodes of temporary blindness lasting one to one and a half minutes, followed by nausea, vomiting, and abdominal pain. His seizures became increasingly complicated over time, leading to periods of widespread bodily rigidity and involuntary tremors. Biting of the tongue, a sign of widespread tonic-clonic seizures, was present during severe episodes. These episodes, which had no known aura and occasionally resulted in unconsciousness, lasted one to three minutes.

The results of the electroencephalogram (EEG) showed aberrant epileptiform activity. One potential contributory factor was identified as a brain injury occurring at the age of one year and six months. He was taking an anti-epileptic medication, which helped to regulate his seizures to some extent. In the past, homeopathic therapy had temporarily reduced the frequency of seizures. But breakthrough seizures continued, especially when doses were missed. One of the medication's side effects was noted to be headaches.

Affected individual IV-9, a female was diagnosed with this disease a female at the age of 25-year-old, with first seizures at the age of 15. Every minute or so, she had abrupt experiences of losing consciousness and awareness, which were followed by total amnesia of the incident. She didn't have any prior head injuries. Her seizures persisted on occasion. She claimed that one of the negative effects was dizziness. The last seizure occurred two and a half months prior to the assessment.

Both affected individuals exhibited generalized seizures beginning in childhood or adolescence, partial medication control, and lacked dysmorphic or syndromic features. No signs of facial dysmorphism, intellectual impairment, or systemic organ involvement were found. A recessive genetic etiology is most likely indicated by the condition's presence in both sexes, consanguinity, and family history.

The clinical features of affected members from Family EP2 are presented in Table 4.2.

Individual ID	V-2A	IV-9
Age at Onset	14 years	25 years
Epilepsy Type	Generalized tonic-clonic	Generalized epilepsy
Seizure Frequency/Features	Initially transient blindness with nausea/vomiting; progressed to tremors, stiffness, and tongue biting; occasional LOC	Sudden loss of awareness and consciousness; amnesia after events
Triggers	Missed doses	Hormonal changes
EEG Findings	Abnormal epileptiform activity	Abnormal EEG consistent with epilepsy
Comorbidities	History of head injury	-
Duration of Seizures	1 minute	1 minute
Current Medication & Dose	Anti-epileptic syrup	Telox, Levitra
Reported Side Effects	headache	Dizziness

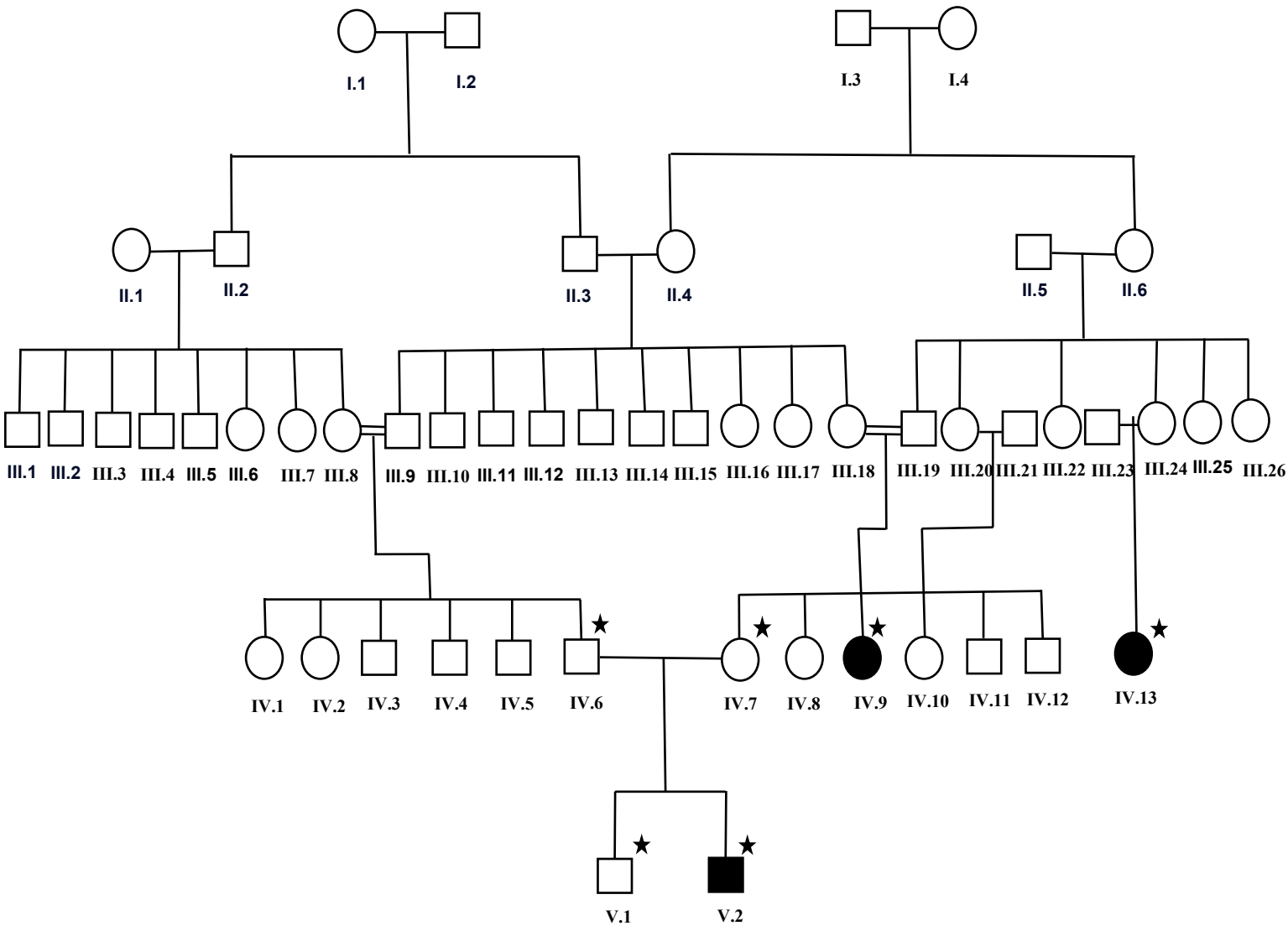

Figure 4.2:- Pedigree of family EP 1 segregating Autosomal recessive Epilepsy The samples were collected from the members that are marked with (*) in the pedigree.

4.3 Sequencing Analysis of genes

There are different genes which are involved in the proper functioning of human brain and variants in these genes can lead to improper functioning and cause severe brain diseases including epilepsy. For variant analysis of one candidate genes SCN1A (sodium voltage-gated channel alpha subunit 1) gene confined on human chromosome 2q24.3 were selected for variant analysis.

4.3.1 Mutation Screening

To find the genetic variation, specific genetic testing was conducted in light of the strong family history pattern. Three particular exons were chosen for Sanger sequencing analysis. based on prior research that links the SCN1A gene's exons 5, 21, and 26 to the pathophysiology of epilepsy.

4.3.2 SCN1A Gene

SCN1A gene was selected for variant analysis in Pakistani epilepsy patients. Earlier studies reported different mutations in the gene. SCN1A gene have 29 exons. Exon 5, 21, and 26 of SCN1A gene is selected for variant analysis. Reference sequence of this selected exon was obtained from Ensemble genome browser (https://asia.ensembl.org/index.html). After analysis of the chromatogram of the exons (exon 5), (exon 21), (exon 26) were done on Bioedit Sequence Alignment tool. no mutation was identified in the selected exon of SCN1A gene. According to the findings. There was no indication of

homozygous or heterozygous alterations, frameshifts, or minor deletions or insertions, and every sequence matched the reference sequence precisely. This finding implies that mutations in the studied exonic regions of the SCN1A gene are not the source of the epileptic phenotype in Family EP1 and EP 2.

Since the screened exons did not include any variants, the underlying mutation may still be present in unscreened exons, intronic regions, regulatory elements, or even distinct genes. The genetic variables in this family may be revealed by future studies using whole-exome sequencing (WES) or whole-genome sequencing (WGS).

5. Discussion

Epilepsy is a long-term neurological condition marked by frequent, unprovoked seizures resulting from abnormal electrical activity in the brain (Singh & Sander, 2020). It significantly impacts global health, especially in low- and middle-income nations where access to diagnostic and treatment options is often insufficient. While environmental influences like injuries, infections, and complications during birth are significant, a considerable number of epilepsy cases are linked to genetic causes.

In Pakistan, the high rates of consanguineous marriages, especially between first cousins, further complicate the situation by increasing the chances of homozygosity for rare recessive alleles. This cultural norm raises the risk of inherited epilepsies and contributes to the significant genetic diversity found within the local population (Khan *et al.,* 2021). Consequently, families affected by these conditions often exhibit differences in the timing of seizure onset, intensity, and types of seizures, even among relatives, which complicates both clinical diagnosis and genetic assessment.

Among the various genes linked to epilepsy, SCN1A has become one of the most important in clinical settings. Variants in the SCN1A gene have been frequently connected to severe conditions like Dravet syndrome and genetic epilepsy associated with febrile seizures plus (GEFS+). These variants generally disrupt sodium channel functionality in inhibitory interneurons, which leads to increased neuronal excitability and a higher likelihood of seizures (Catterall, 2018; Ogiwara *et al.,* 2007). Nonetheless, even with its strong association, SCN1A does not explain all familial cases of epilepsy, highlighting the multifactorial and diverse nature of the disorder.

In this study, the two Pakistani families with known history of epilepsy, the exons 5, 21, and 26 of the SCN1A gene were subjected to sanger sequencing. After the analysis of Sanger sequencing result no pathogenic variants in the targeted regions were identified. These results suggest that the examined SCN1A exons do not contribute to epilepsy in these families, thus emphasizing the idea of considerable genetic diversity in epilepsy.

The lack of mutations in the targeted exons highlights several key points. Although SCN1A mutations account for approximately 70–80% of Dravet syndrome cases worldwide (Claes *et al.,* 2001; Marini *et al.,* 2011), studies based on populations show varying mutation rates across different ethnic groups. In

South Asian and Middle Eastern communities, a notably lower incidence of SCN1A mutations has been observed, indicating that other genes might have a more substantial influence in these areas (Abdulkareem *et al.,* 2023). Likewise, research in Pakistan has uncovered new variants in additional candidate genes such as CNTNAP2, SPTBN5, CARS2, and CLCN4 among consanguineous epilepsy families (Yasin *et al.,* 2024; Younus *et al.,* 2020). These results reinforce the idea that while SCN1A is still clinically significant, it cannot be regarded as the only genetic marker for epilepsy in Pakistan.

There are several potential reasons for the lack of mutations found in this study. Pathogenic variants might be in unexamined exons, intronic areas, or regulatory elements of SCN1A (Parihar & Ganesh, 2013). Another possibility is that other ion channel and synaptic genes like SCN2A, KCNQ2, or GABRA1 may contain causative variants (Hirose, 2014; Liao *et al.,* 2010). Additionally, the epilepsy observed in these families could be attributed to a polygenic mechanism, where several low-impact variants work together with environmental factors such as perinatal stress or head trauma (Ottman *et al.*, 2010). Lastly, we must also consider methodological limitations: while Sanger sequencing is reliable, it is limited to specific regions and cannot identify copy number variations, significant deletions, or deep intronic mutations (Sanger *et al.,* 1977).

Despite the negative results, this research contributes significant insights into the genetics of epilepsy within Pakistan. From a clinical standpoint, it highlights the necessity of utilizing more extensive molecular approaches, such as whole-exome sequencing (WES) or whole-genome sequencing (WGS), which can identify a broader range of variants, including structural alterations. On a public health level, it is essential to combine genetic testing with counseling initiatives in communities with high rates of consanguinity, as this could enable early identification, assist in treatment choices, and support reproductive planning efforts (Hamamy, 2012; Riaz *et al.,* 2019).

Despite these findings, the study has multiple limitations. Firstly, the analysis focused solely on three exons of SCN1A, which means much of the gene remains unexamined. Secondly, the limited sample size of just two families constrains the broader applicability of the results. Lastly, additional candidate genes linked to epilepsy were not explored, and no functional validation was conducted.

Future studies should concentrate on extensive, population-centric research employing WES or WGS to identify new variants, subsequently validated through in silico and in vitro techniques. Expanding

research across various Pakistani populations will improve the identification of distinct genetic factors and elucidate their involvement in the development of epilepsy (Rehman et al., 2022).

In conclusion, the results indicate that no pathogenic variants were detected in exons 5, 21, or 26 of the SCN1A gene among the two families with epilepsy from Pakistan that were examined. These findings support the idea that familial epilepsies in Pakistan could be influenced by a wider range of genetic factors beyond SCN1A, highlighting the genetic diversity inherent in the disorder. To enhance the accuracy of diagnoses, facilitate treatment strategies, and alleviate the impact of the disease through improved genetic counseling, it will be crucial to conduct more extensive genetic research in this population.

6. References

Abdulkareem, A. A., Zaman, Q., Khan, H., Khan, S., Rehman, G., Tariq, N., … Jelani, M. (2023). Whole exome sequencing identified five novel variants in CNTN2, CARS2, ARSA, and CLCN4 leading to epilepsy in consanguineous families. *Frontiers in Genetics, 14*, 1185065.

Ahmad, F., Shabbir, R., & Jafri, S. I. (2021). Genetic counseling and awareness in consanguineous populations: A need for culturally sensitive approaches. *Journal of Community Genetics, 12*(3), 407–419.

Ali, A., Ullah, S., & Shah, S. M. H. (2023). Epidemiological nalysis of epilepsy prevalence in the Pashtun population: A comparative study in Khyber Pakhtunkhwa, Pakistan. *International Journal of Applied and Clinical Research, 1*(01), 13–20.

Baig, S. M., Hamid, Z., Mir, A., et al. (2016). Genetic research challenges in Pakistan: A focus on neurological disorders. *Pakistan Journal of Neurological Sciences, 11*(2), 25–30.

Bamshad, M. J., Ng, S. B., Bigham, A. W., et al. (2011). Exome sequencing as a tool for Mendelian disease gene discovery. *Nature Reviews Genetics, 12*(11), 745–755.

Bashir, M. A., & Khalid, A. (2020). Misconceptions in epilepsy and its differentiation from psychogenic nonepileptic events in Pakistan: The dilemma in underdeveloped regions. *Seizure, 81*, 259–261.

Baulac, S., et al. (2001). First genetic evidence of GABA(A) receptor dysfunction in epilepsy: A mutation in the gamma2-subunit gene. *Nature Genetics, 28*(1), 46–48.

Bener, A., et al. (2006). Consanguineous marriages and their effects on common adult diseases: Studies from Qatar. *Annals of Saudi Medicine*.

Berg, A. T., et al. (2010). Revised terminology and concepts for organization of seizures and epilepsies: Report of the ILAE Commission on Classification and Terminology, 2005–2009. *Epilepsia, 51*(4), 676–685.

Ben-Shalom, R., Keeshen, C. M., Berrios, K. N., An, J. Y., Sanders, S. J., & Bender, K. J. (2017). Opposing effects on NaV1.2 function underlie differences between SCN2A variants observed in individuals with autism spectrum disorder or infantile seizures. *Biological Psychiatry, 82*(3), 224–232.

Brackenbury, W. J., & Isom, L. L. (2011). Na channel β subunits: Overachievers of the ion channel family.

Browne, D. L., Gancher, S. T., Nutt, J. G., Brunt, E. R., Smith, E. A., Kramer, P., & Litt, M. (1994). Episodic ataxia/myokymia syndrome is associated with point mutations in the human potassium channel gene KCNA1.

Carvill, G. L., Regan, B. M., Yendle, S. C., O'Roak, B. J., Lozovaya, N., Bruneau, N., … Scheffer, I. E. (2013). GRIN2A mutations cause epilepsy-aphasia spectrum disorders.

Cascino, G. D. (1994). Epilepsy: Contemporary perspectives on evaluation and treatment. *Mayo Clinic Proceedings, 69*, 1199–1211.

Catterall, W. A. (2018). Dravet syndrome: A sodium channel interneuronopathy. *Current Opinion in Physiology, 2*, 42–50.

Claes, L., Del-Favero, J., Ceulemans, B., Lagae, L., Van Broeckhoven, C., & De Jonghe, P. (2001). De novo mutations in the sodium-channel gene SCN1A cause severe myoclonic epilepsy of infancy.

Cossette, P., Liu, L., Brisebois, K., Dong, H., Lortie, A., Vanasse, M., … Rouleau, G. A. (2002). Mutation of GABRA1 in an autosomal dominant form of juvenile myoclonic epilepsy.

Depienne, C., et al. (2009). Spectrum of SCN1A gene mutations associated with Dravet syndrome: Analysis of 333 patients. *Journal of Medical Genetics, 46*(3), 183–191.

Endele, S., Rosenberger, G., Geider, K., Popp, B., Tamer, C., Stefanova, I., … Wieczorek, D. (2010). Mutations in GRIN2A and GRIN2B encoding regulatory subunits of NMDA receptors cause variable neurodevelopmental phenotypes. *Nature Genetics, 42*(11), 1021–1026.

Escayg, A., & Goldin, A. L. (2010). Sodium channel SCN1A and epilepsy: Mutations and mechanisms. *Epilepsia, 51*(9), 1650–1658.

Escayg, A., MacDonald, B. T., Meisler, M. H., Baulac, S., Huberfeld, G., An-Gourfinkel, I., … Malafosse, A. (2001). Mutations of SCN1A, encoding a neuronal sodium channel, in two families with GEFS+.Epi4K Consortium. (2013). De novo mutations in epileptic encephalopathies. *Nature, 501*(7466), 217–221.

Fisher, R. S., et al. (2014). ILAE official report: A practical clinical definition of epilepsy. *Epilepsia, 55*(4), 475–482.

Fisher, R. S., et al. (2017). Instruction manual for the ILAE 2017 operational classification of seizure types. *Epilepsia, 58*(4), 531–542.

Guo, F., Xu, X., Cai, J., Hu, H., Sun, W., & Li, H. (2008). Upregulation of sodium channel subunits Nav1.1, Nav1.3 and β1 in the hippocampus of epileptic rats highlighting ion channel alterations in epilepsy.

Hauser, W. A., & Hesdorffer, D. C. (1990). Epilepsy: Frequency, causes, and consequences. Demos Publications.

Helbig, I., et al. (2008). Genetic determinants of idiopathic generalized epilepsies. *Epilepsia*.

Helbig, I., et al. (2009). 15q13.3 microdeletions increase risk of idiopathic generalized epilepsy. *Nature Genetics, 41*(2), 160–162.

Helbig, I., & Ellis, C. A. (2020). The genetic landscape of epilepsy. *Current Opinion in Genetics & Development, 65*, 1–8.

Hildebrand, M. S., et al. (2013). Recent advances in the molecular genetics of epilepsy. *Journal of Medical Genetics, 50*, 271–279.

Howell, K. B., McMahon, J. M., Carvill, G. L., Tambunan, D., Mackay, M. T., Rodriguez-Casero, V., … Scheffer, I. E. (2015). SCN2A encephalopathy: A major cause of epilepsy of infancy with migrating focal seizures. *Neurology, 85*(11), 958–966.

Imbrici, P., Camerino, D. C., & Tricarico, D. (2006). Major channels involved in neuropsychiatric disorders and therapeutic perspectives. *CNS & Neurological Disorders – Drug Targets, 5*(6), 675–682.

International League Against Epilepsy Consortium on Complex Epilepsies. (2018). Genome-wide mega-analysis identifies 16 loci and highlights diverse biological mechanisms in the common epilepsies. *Nature Communications, 9*(1), 5269.

Jalili, I. K. (2011). Consanguinity. [Cited March 27, 2011]. Available from: [link].

Kalachikov, S., et al. (2002). Mutations in LGI1 cause autosomal-dominant partial epilepsy with auditory features. *Nature Genetics, 30*(3), 335–341.

Khan, A., Ali, S., & Rehman, A. (2020). Challenges in genetic research and healthcare in developing countries: A Pakistani perspective. *Human Genomics, 14*(1), 34.

Khan, S., et al. (2021). Consanguinity and autosomal recessive disorders: A study in Pakistani families. *BMC Medical Genetics*.

Khatri, I. A., et al. (2003). Epidemiology of epilepsy in Pakistan: Review of literature. *Journal of the Pakistan Medical Association, 53*(12), 594–597.

Lemke, J. R., et al. (2012). Targeted next-generation sequencing in epileptic encephalopathies. *JAMA Neurology, 69*(4), 474–481.

Lesca, G., Rudolf, G., Bruneau, N., Lozovaya, N., Labalme, A., Boutry-Kryza, N., … Szepetowski, P. (2013). GRIN2A mutations in acquired epileptic aphasia and related childhood focal epilepsies and speech disorders.

Lindy, A. S., et al. (2018). Diagnostic outcomes for genetic testing of 70 genes in 7,812 patients with epilepsy and neurodevelopmental disorders. *Epilepsia, 59*(5), 1062–1071.

Liao, Y., Anttonen, A. K., Liukkonen, E., Gaily, E., Maljevic, S., Schubert, S., … Lehesjoki, A. E. (2010). SCN2A mutation associated with neonatal epilepsy, late-onset episodic ataxia, myoclonus, and pain. *Neurology, 75*(16), 1454–1458.

Macdonald, R. L., Kang, J. Q., & Gallagher, M. J. (2010). Mutations in GABAA_AA receptor subunits associated with genetic epilepsies. *Journal of Physiology, 588*(11), 1861–1869.

Masnada, S., Hedrich, U. B. S., Gardella, E., Schubert, J., Kaiwar, C., Klee, E. W., … Lerche, H. (2017). Clinical spectrum and genotype–phenotype associations of KCNA2-related encephalopathy. *Brain, 140*(9), 2337–2354.

McDonald-McGinn, D. M., et al. (2015). 22q11.2 deletion syndrome. *Nature Reviews Disease Primers, 1*, 15071.

Mefford, H. C., et al. (2008). Recurrent rearrangements of chromosome 1q21.1 and variable pediatric phenotypes. *New England Journal of Medicine, 359*(16), 1685–1699.

Meng, H., Xu, H. Q., Yu, L., Lin, G. W., He, N., Su, T., … Liao, W. P. (2015). The SCN1A mutation database: Updating information and analysis of the relationships among genotype, functional alteration, and phenotype. *Human Mutation, 36*(6), 573–580.

Modell, B., & Darr, A. (2002). Genetic counselling and customary consanguineous marriage. *Nature Reviews Genetics, 3*(3), 225–229.

Mogal, Z., & Aziz, H. (2020). Epilepsy treatment gap and stigma reduction in Pakistan: A tested public awareness model. *Epilepsy & Behavior, 102*, 106637.

Monica, R. Family history of epilepsy. [Cited September 16, 2011]. Available from: http://www.revolutionhealth.com/conditions/brain-nerves/epilepsy/family-history

Nasir, R., Fatima, A., & Rehman, A. (2021). Genetic epidemiology of epilepsy in consanguineous populations. *Journal of Neurogenetics, 35*(2), 87–98.

O'Malley, H. A., & Isom, L. L. (2015). Sodium channel β subunits: Emerging targets in channelopathies. *Annual Review of Physiology, 77*, 481–504.

Ottman, R., et al. (1996). Clinical indicators of genetic susceptibility to epilepsy. *Epilepsia, 37*, 353–361.

Ottman, R., et al. (2010). Genetic testing in the epilepsies—Report of the ILAE Genetics Commission.

Ogiwara, I., Miyamoto, H., Morita, N., Atapour, N., Mazaki, E., Inoue, I., … Yamakawa, K. (2007). NaV1.1 localizes to axons of parvalbumin-positive inhibitory interneurons: A circuit basis for epileptic seizures in mice carrying an Scn1a mutation.

Paoletti, P., Bellone, C., & Zhou, Q. (2013). NMDA receptor subunit diversity: Impact on receptor properties, synaptic plasticity and disease.

Parihar, R., & Ganesh, S. (2013). The SCN1A gene variants and epileptic encephalopathies. *Journal of Human Genetics, 58*(9), 573–580.

Patino, G. A., & Isom, L. L. (2010). Electrophysiology and beyond: Multiple roles of Na+ channel β subunits in development and disease.

Patino, G. A., Claes, L. R. F., Lopez-Santiago, L. F., Slat, E. A., Dondeti, R. S., Chen, C., … Isom, L. L. (2009). A functional null mutation of SCN1B in a patient with Dravet syndrome.

Platzer, K., Yuan, H., Schütz, H., Winschel, A., Chen, W., Hu, C., … von Stülpnagel, C. (2017). GRIN2A- and GRIN2B-related neurodevelopmental disorders: Linking N-methyl-D-aspartate receptor function to clinical phenotype.

Riaz, M., Lodhi, A., Khan, F., & Lacaze, P. (2019). Implementation of public health genomics in Pakistan. *European Journal of Human Genetics, 27*(10), 1485–1492.

Rehman, A. U., Morell, R. J., & Friedman, T. B. (2015). Genetics of hearing loss in consanguineous families. *Current Opinion in Genetics & Development, 33*, 232–240.

Saleem, T., et al. (2021). Mutational analysis of Myoclonin1 (EFHC1) gene in Pakistani juvenile myoclonic epilepsy patients. *Biomed Research International, 2021*, Article ID 7509825.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, 74*(12), 5463–5467.

Scheffer, I. E., Harkin, L. A., Grinton, B. E., Dibbens, L. M., Turner, S. J., Zielinski, M. A., … Berkovic, S. F. (2007). Temporal lobe epilepsy and GEFS+ phenotypes associated with SCN1B mutations. *Brain, 130*(1), 100–109.

Sheikh, S., Nasir, R., & Yousaf, S. (2019). Genetic testing in epilepsy: Current status and future directions in Pakistan. *Epilepsy & Behavior Reports, 13*, 100352.

Shi, X., Yasumoto, S., Kurahashi, H., Nakagawa, E., Fukasawa, T., Uchiya, S., … Yamakawa, K. (2012). Clinical spectrum of SCN2A mutations. *Brain and Development, 34*(7), 541–545.

Singh, G., & Sander, J. W. (2020). The global burden of epilepsy report: Implications for low- and middle-income countries. *Epilepsy & Behavior, 105*, 1069.

Singh, N. A., et al. (1998). A novel potassium channel gene, KCNQ2, is mutated in an inherited epilepsy of newborns. *Nature Genetics, 18*(1), 25–29.

Syrbe, S., Hedrich, U. B. S., Riesch, E., Djemie, T., Müller, S., Møller, R. S., … Lerche, H. (2015). De novo loss- or gain-of-function mutations in KCNA2 cause epileptic encephalopathy. *Nature Genetics, 47*(4), 393–399.

Tadmouri, G. O., Nair, P., Obeid, T., Al Ali, M. T., Al Khaja, N., & Hamamy, H. A. (2009). Consanguinity and reproductive health among Arabs. *Reproductive Health, 6*, 17.

Thomas, R. H., & Berkovic, S. F. (2014). The hidden genetics of epilepsy—a clinically important new paradigm. *Nature Reviews Neurology, 10*(5), 283–292.

Wallace, R. H., et al. (1998). Mutant GABRA1 subunit in a family with idiopathic generalized epilepsy. *Nature Genetics, 19*, 235–238.

Weckhuysen, S., Holmgren, P., Hendrickx, R., Jansen, A. C., Hjalgrim, H., Lesca, G., … De Jonghe, P. (2012). KCNB1 encephalopathy: A new neurodevelopmental disorder characterized by epilepsy and developmental delay. *Brain, 135*(6), 1741–1751.

Zhang, Y., Pak, C., Han, Y., Ahlenius, H., Zhang, Z., Chanda, S., … Südhof, T. C. (2013). Rapid single-step induction of functional neurons from human pluripotent stem cells. *Neuron, 78*(5), 785–798.