In vitro identification of CACN1h 9 axonal SNP in epileptic patients

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Abstract

Childhood absence epilepsy (CAE) is an autosomal dominant disorder and a heterogeneous familial condition in which family members express absence seizures initially, and then show multiple phenotypes of myoclonic epilepsy, including partial or absence seizures and generalized tonic conic seizures. The background of this study was that the world health organization (WHO) reported that neurological disorders affect one billion people worldwide, including 50 million affected by epilepsy. Various parameters were used in the present study. It was aimed at investigating the coding regions of CACNA1H gene for analyzing the mutations involved in epilepsy. Blood samples of an unrelated true representative of CAE were collected from psychiatry departments of different hospitals in Lahore. DNA was extracted with the standard protocol and amplifications of the CACNA1H regions were done with specially designed primers. Later on, analysis of the results was done by sequencing of target fragments was carried out. Sequences were
analyzed through BioEdit software and then aligned with the help of clustalW2 software. It has been identified by the recent study on the absence of epileptic patients in Pakistan that the gene CACNA1h has an SNP in exon 9 at the position (2025G>A) which eventually alters the protein, making it hyperactive as the mutations are in the sensor regions of the protein, thus giving a ‘gain in function’ property to the ion channel. In conclusion, there is a need to explore the site of the gene along with other gene mutations causing epilepsy in the local population of Punjab and Pakistan. This will ultimately help to develop genetic counseling strategies, gene therapies, and prenatal diagnostic procedures for the population of Pakistan.

Keywords: Epilepsy, DNA amplification, Sequences, mutations, and prenatal diagnostic

INTRODUCTION

The disease epilepsy is first discovered by a Greek physician Hippocrates in 400 B.C and he wrote in his book “on the sacred disease” and he proved it to be a brain disorder (Steven et al. 2004). The epilepsy term has been derived from the Greek language “epilambanein” which means to seize or to attack. Epileptic seizures are recurrent attacks, short lasting, brief jerks of altered consciousness and motor activity (Seyfried et al. 2005). Epilepsy is an ailment of the nervous system that is characterized by onward development of seizures and by cognitive, psychological, neurobiological and social problems. According to WHO estimates, approximately eight people per 1000 population in the world have this disease. The commonness of this ailment is higher in underdeveloped countries as compared to developed states. Most of the people, approximately half of the “50 million” with this neurological defect, epilepsy, are inhabitants of Asia. Although most of the research is planned and performed in Asia, there is less authentic documentation about the burden of the disease. In 1998, Jallon wrote a report on these studies from Asia, which were mostly completed in the late 1980s, and gave the conclusions that the disease is prevalent in Asia, ranging from 1.5 per 1000 in Japan to 10.0 per 1000 in Pakistan. (Epidemiology, aetiology, and clinical management of epilepsy in Asia). During the attack of a seizure, epileptic victims often jerk vigorously and sometimes their extremities lose consciousness. On the other hand, epileptic patients may display emotional outbursts or periods of confusion. Generalized seizures are those seizures that involve the entire body and are classified into two forms, generally known as tonic-clonic (grand mal) and absence seizures (petit mal). Partial seizures are those when just a part of
the body is affected. Some cases of epilepsy are inherited, but most are not. This is known from a combination of epidemiology and in vitro research that defects in single genes or a combination of genes, exacerbated by environmental factors, can lead to epilepsy. (Current Opinion in Neurology 1998;11:123-27). These epileptic problems mainly arise in children under the age of 8 months and are characterised by focal seized diseases that normally occur in clusters. The clinical pathway is usually harmless, with spontaneous resolution within 2 years and in most cases normal development of the psychomotor. Focal epileptics may be described as benign neonatal family seizures (BFNS), benign family newborn infant seizures (BFNIS) or benign family infant seizures (Benign) during the first year of life based on the age of development (BFIS)(Scala, Bianchi et al. 2020).

Epilepsy influences all age groups but for children, a variety of issues exist that can have bad impacts on one's childhood (Epilepsy: page 247-48). There are some types of idiopathic generalized as well as some specific types of epilepsy which follow an autosomal dominant, monogenic (or mendelian) pattern of inheritance, which will increase the likelihood of identification of the affected gene. These mutations have been found in Ca, Cl, K, and Na channels, adding to the list of channelopathies which influence excitable tissues (Robinson and Gardiner, 2000). In general, consistent with the notion that epilepsy is a disorder of hyperexcitability, the majority of mutations display gain-of-function characteristics such as hyperpolarizing shifts in activation thresholds and increased current density. Many of the mutations are clustered around the intracellular linker connecting transmembrane segments S2 and S3 in domain I and are thought to affect surface expression of the channel. While these mutations alone may not be sufficient to induce an epileptic phenotype, they could increase excitability in a manner that contributes towards the generation or propagation of seizures (Zamponi G et al 2009). Molecular genetics has revolutionized the understanding of epilepsy genetics as genetic differences are responsible for heritable variation among individuals, including disease susceptibility (Hedera et al. 2011). Most CAE associated genes include gene of ion channel genes, like calcium channel, GABA receptor, acetylcholine receptor etc. CAE is closely linked to the calcium channel genes CACNA1H and CACNG3. (Yalçın 2012).

CACNA1H is the most important ion channel that acts on ligand-gated calcium channels in the mammalian brain. The GABAA receptor is trimeric as it consists of three subunits namely alpha
(α), beta (β) and gamma (γ). The Alpha subunit is encoded by the CACNA1H gene. Any polymorphism in this gene may contribute to the susceptibility to CAE.

The CACNA1H gene in humans is located on chromosome no. 16 and is mapped to 16p36.33 (Figure 1) www.ncbi.nlm.nih.gov, flanked by PRKCZ (176982) and KIAA1751 (Figure 2) www.ncbi.nlm.nih.gov). This gene contains the sequence of an 11,825 bp region from base 1950768 to 1962192 bp. It consists of nine exons.

![Figure 1: CACNA1H gene locus on Chromosome 16 (genecards)](image)

Chromosome 16

![Figure 2: Map of CACNA1H gene (NCBI)](image)

The present study was conducted to find out the mutations in the CACNA1H gene in CAE patients from Punjab.

The purpose of this study is to evaluate the prevalence of CACNA1H gene mutations in sensor region of exon 9a among CAE patients in Lahore with the help of literature review and analysis of collected data results.

**Methodology**

**Selection of Individuals and Clinical Evaluation**
First of all, individuals and families that have the neurological disorder CAE were searched out and identified. Patients were selected according to the clinical information provided by experienced neurologist. All available patients were interviewed and seizure/epilepsy histories were documented by asking relevant questions about individual relations, medical history or all important information related to the disorder, to minimize the presence of other abnormalities and environmental causes for the disorder. Written informed consent was obtained from all affected adults.

A total of 12 patients with CAE were identified from the pediatrics and the psychiatry departments of different hospitals in Lahore (Sir Ganga Ram Hospital and Children Hospital complex) and were enrolled in the present study. All patients were less than 12 years age. The families of enrolled patients agreed to take part in the present study and were allowed to collect blood samples (3-5 mL) of affected individuals. A consent form was signed by the parents or responsible adult. After DNA extraction, four primer sets were designed and synthesized to amplify the exons 9-11 of CACNA1H gene.

**Blood Sampling (Collection and Storage)**

Blood samples were drawn from 14 patients by an experienced technician into a 5mL Vacutainer tube containing ethylenediamine tetra-acetic acid (EDTA) (0.5M), as an anticoagulant. The patient’s name and code were written on the side of the tube. A list of all samples was prepared, after reaching the Molecular Cytogenetic and Genomics Laboratory at the Institute of Biochemistry and Biotechnology, UVAS-Lahore. The samples were then stored (at -20° C) for further processing.

**DNA Extraction**

The following materials and methods were used for DNA extraction and PCR amplification used by Singer *et al.*, 1988 with slight optimization and modifications.

**DNA Quantification**
After extraction, quantification and analysis of DNA quality are mandatory to determine the approximate concentration of DNA obtained for further exploration. DNA quantification was carried out by gel electrophoresis and by spectrophotometric analysis.

**Primer Designing**

Primers were designed with the help of Primer3 software (http://frodo.wi.mit.edu/primer3/), by using the sequence information available at the NCBI website (www.ncbi.nlm.nih.gov) about the human CACNA1H gene (Accession # NC_000001.10) found on chromosome 16. The exon primed intron-centred (EPIC) approach was used to generate specific amplicons.

The primers were synthesized as unlabelled from e-oligos.

| Sr. No. | Primer Name | Length (bp) | Primer Sequence (5'-3') | TM | Product Size (bp) |
|---------|-------------|-------------|-------------------------|----|------------------|
| 1       | CACNA1Hex9 Forward | 20 | CATCTCAGAGGCAACCATGG | 57.0 | 450 |
|         | CACNA1Hex9 Reverse  | 20 | GACCTTGGAGCGGATGTTTC | 57.4 |     |
| 2       | CACNA1Hex9 Forward | 18 | GGGCAAAACACAGTCTGAG | 54.7 | 463 |
|         | CACNA1Hex9 Reverse  | 19 | GACTGCTCCAGGCTTCTG  | 57.3 |     |

Primer sequences were optimized using the online tool, OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html). The specificity of the primers was checked by the insilico PCR online tool available at the University of California, Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu/cgi-bin/hgPcr)
PCR Amplification

Both forward and reverse primers were used to amplify 14 DNA samples. PCR was carried out in four steps by the use of a thermocycler.

Gel Electrophoresis

Amplification was confirmed by agarose gel electrophoresis. To check the PCR product 1.2% agarose gel was prepared by using 1.2 g of agarose. The DNA ladder of 1Kb was sued as molecular marker. The gel was viewed under UV light in Gel documentation system (Bio-Rad) and photographed. Then the PCR Products was precipitated for Sequencing.

Sequencing of PCR Products

After the precipitation of PCR, products were sequenced. Sequencing was done on the principle of Sanger’s chain termination method.

Precipitation of Sequencing Samples

PCR products, after sequencing reactions were precipitated with ethanol and loaded onto the ABI PRISM 3130 Sequencer genetic analyzer according to manufacturer’s instructions given in the technical manuals. The ABI PRISM sequencing analysis software (v. 3.7) was used to analyze the samples after each run.

Analysis of Sequencing Samples

Analysis of the sequences was done with the help of appropriate Bioinformatics software and tools. Sequences were analyzed manually by using BioEdit software version (V.7.0). Nucleotide BLAST program, available at NCBI website (http://www.ncbi.nlm.nih.gov/BLAST) was used for sequencing homology searches in public databases (Altschul et al. 1990). The sequences were also BLAST against the reported reference sequence. The polymorphism was detected from the sequences by multiple sequence alignment using ClustalW2 online tool available at EBI (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Any change in the DNA sequence was confirmed by sequencing both sense and antisense strands. Restriction analysis was also performed for
mutated sequences using the NEBcutter online tool available at BioLabs (http://tools.neb.com/NEBcutter2/).

RESULTS

PCR Amplification of Exon 9

Figure 3.1 showing the PCR amplification of CACNA1H gene’s exon 4. This exon was amplified using the CACNA1H ex9 primer set by a touchdown PCR reaction giving a range of annealing temperature (60°C-50°C). To check the required amplicon, gel (1.2 %) was used to run the samples and a 1 kb ladder. The product size for this primer set was 450 bp.

Lane M: Marker 1kb SM0313 (Fermentas)

Lane 1-14: Amplified fragments of CACNA1H Exon 9 isolated from patients with CAE

Product size: 450bp
PCR Amplification of Exon 9

Figure 3.2 showing the PCR amplification of axon 9 of the gene. This exonic region was amplified using CACNA1H primer set by following the touchdown PCR protocol. The annealing temperature range was 60-50°C. The product size for this primer (463 bp) was confirmed for the amplicons by gel electrophoresis.

Lane M: Marker 1kb SM0313 (Fermentas)

Lane 1-14: Amplified fragments of CACNA1H Exon 9 isolated from patients with CAE

Product size: 463 bp

Sequencing of PCR Products

After the amplification of the desired regions of the DNA, PCR products were precipitated and loaded onto a 1.2% gel to see the concentration of DNA per µL. The purpose of precipitation was to eliminate the nonspecific products from the amplified sample. These precipitated products were then sequenced for mutational analysis.

The present study was aimed at genetically characterizing the CACNA1H gene to identify mutations associated with CAE. Primers designed for the amplification were based on the EPIC approach. Four amplification and sequencing primer sets were used for the analysis of mutation in exon 9.
A series of 12 unrelated patients with CAE were screened for mutation in the CACNA1H gene. No mutation was found in exon 9a. While already reported, mutations in the exonic sequence of CACNA1H gene were found in 5 out of 14 CAE patients. These changes were observed in a PCR fragment amplified by primer 2 in the region of 9th exon. Subsequent analysis of these fragments identified transition mutations (2025G>A) in exons 9 (Figure 5).

Figure 4: Chromatograph from exon 9a

Mutation found in the patients with CAE

Results of SNP in Exon 9
Figure 6 (a): Chromatograph from exon 9a (control)

Figure 6 (b): Chromatograph from exon 9b
Figure 6 (c): Chromatograph from exon 9b

Results of BLAST

| Query | Sbjct |
|-------|-------|
| 86    | 243   |
| 146   | 183   |
| 206   | 123   |
| 266   | 63    |
| 326   | 3     |
| 76    | 143   |
| 246   | 173   |
| 106   | 1     |

```plaintext
| Query | Sbjct |
|-------|-------|
| TGTGAATTTACGTGGCTTCACTGTAAGAGAAACATAGAAAATGTGGCACATAGAGAGG | TGTGAATTTACGTGGCTTCACTGTAAGAGAAACATAGAAAATGTGGCACATAGAGAGG |
| TGCAAGATTTTATTTTACTTGGCTTCACTGTAAGAGAAACATAGAAAATGTGGCACATAGAGAGG | TGCAAGATTTTATTTTACTTGGCTTCACTGTAAGAGAAACATAGAAAATGTGGCACATAGAGAGG |
| AGTAATTATTACACTATTTTGGTACCAGTTACTAATTTCACCAAGAGACCAAGCCAGCATAGTAGAG | AGTAATTATTACACTATTTTGGTACCAGTTACTAATTTCACCAAGAGACCAAGCCAGCATAGTAGAG |
| TTTGTTTTCAAAGAAATATCTTTTTTAAGTATGCACATGCACCAAGCACGCATAGTAGAG | TTTGTTTTCAAAGAAATATCTTTTTTAAGTATGCACATGCACCAAGCACGCATAGTAGAG |
| TTT | TTT |
| TGTGAATTTACGTGGCTTCACTGTAAGAGAAACATAGAAAATGTGGCACATAGAGAGG | TGTGAATTTACGTGGCTTCACTGTAAGAGAAACATAGAAAATGTGGCACATAGAGAGG |
| TGCAAGATTTTATTTTACTTGGCTTCACTGTAAGAGAAACATAGAAAATGTGGCACATAGAGAGG | TGCAAGATTTTATTTTACTTGGCTTCACTGTAAGAGAAACATAGAAAATGTGGCACATAGAGAGG |
| AGTAATTATTACACTATTTTGGTACCAGTTACTAATTTCACCAAGAGACCAAGCCAGCATAGTAGAG | AGTAATTATTACACTATTTTGGTACCAGTTACTAATTTCACCAAGAGACCAAGCCAGCATAGTAGAG |
| TTTGTTTTCAAAGAAATATCTTTTTTAAGTATGCACATGCACCAAGCACGCATAGTAGAG | TTTGTTTTCAAAGAAATATCTTTTTTAAGTATGCACATGCACCAAGCACGCATAGTAGAG |
| TTT | TTT |
| TGTGAATTTACGTGGCTTCACTGTAAGAGAAACATAGAAAATGTGGCACATAGAGAGG | TGTGAATTTACGTGGCTTCACTGTAAGAGAAACATAGAAAATGTGGCACATAGAGAGG |
| TGCAAGATTTTATTTTACTTGGCTTCACTGTAAGAGAAACATAGAAAATGTGGCACATAGAGAGG | TGCAAGATTTTATTTTACTTGGCTTCACTGTAAGAGAAACATAGAAAATGTGGCACATAGAGAGG |
| AGTAATTATTACACTATTTTGGTACCAGTTACTAATTTCACCAAGAGACCAAGCCAGCATAGTAGAG | AGTAATTATTACACTATTTTGGTACCAGTTACTAATTTCACCAAGAGACCAAGCCAGCATAGTAGAG |
| TTTGTTTTCAAAGAAATATCTTTTTTAAGTATGCACATGCACCAAGCACGCATAGTAGAG | TTTGTTTTCAAAGAAATATCTTTTTTAAGTATGCACATGCACCAAGCACGCATAGTAGAG |
| TTT | TTT |
```
| Sbj ct  | AGTAATTTATTCACAATCTAATTTGGTACCAGTTACTAATTCACCACATTTGAATAACTAT | 74 |
|--------|---------------------------------------------------------------|----|
| Query  | TTTGTTTTTCAAAAGAAATATCTTTTGAATATCGCATGCACCATAGAGAG       | 225|
| Sbj ct  | TTTGTTTTTCAAAAGAAATATCTTTTGAATATCGCATGCACCATAGAGAG       | 6  |

| Query  | TTTT | 3  |
|--------|------|----|
| Sbj ct  | TTTGTTTTTCAAAAGAAATATCTTTTGAATATCGCATGCACCATAGAGAG       | 6  |
| Query  | TTTT | 4  |
| Sbj ct  | TTTGTTTTTCAAAAGAAATATCTTTTGAATATCGCATGCACCATAGAGAG       | 4  |

**Figure 7 Results of Blast**: Sense strand shows the normal sequence. Subject strand has mutation at position 2025 where A is mutated by G (2025G>A) as highlighted in yellow colour. These mutations are in sensor region of exon 9a giving the gain in function property.

**DISCUSSION**

A set of primers with product size of 450bp and 463bp are used for the amplification of CACNA1H gene’s exon 4 by following the touchdown PCR protocol. After the amplification of the desired regions of the DNA, PCR products were precipitated. These precipitated products were then sequenced for mutation analysis. Four amplification and sequencing primer sets were used for the analysis of mutation in exon 9a that were based on the EPIC approach. There was no significant mutation found in exon 9a in the participants that were not the victim of the CAE. Previously reported CAE patient’s mutant genes were observed in a PCR fragment amplified by primer 2 in the region of 9th exon. Subsequent analysis of these fragments identified transition mutations (2025G>A) in exons 9. Childhood absence epilepsy (CAE) is an autosomal dominant disorder and a heterogeneous familial condition in which family members express absence seizures initially, and then show multiple phenotypes of myoclonic epilepsy, including partial or absence seizures and generalized tonic conic seizures (Caplan R, et al, 2008) Sequences were analyzed through BioEdit software and then aligned with the help of clustalW2 software. It has been identified by the recent study on the absence of epileptic patients in Pakistan that the gene CACNA1h has an SNP in exon 9 at the position (2025G>A) which eventually alters the protein, making it
hyperactive as the mutations are in the sensor regions of the protein, thus giving a ‘gain in function’ property to the ion channel (Aziz et al, 1994)

In literature, many other such exonic and intronic mutations in CACNA1H receptor genes have also been reported to be involved in epilepsy susceptibility. These mutations drop the CACNA1H expression level resulting in gain in Ca\textsuperscript{2+} and gain of function of CACNA1H receptors by accelerated activation. CACNA1H polymorphism, (2025G>A) and (4867 G>T) in the exons 9 and 10, was reported to be involved in epilepsy susceptibility and drug resistance (Kumari et al. 2010). The existence of 12 missense mutations in CACNA1H gene was determined by a mutational investigation of Han Chinese population of CAE patients. (Chen, Lu et al. 2003). Comparison of CACNA1H genetic variation with childhood epilepsy absence. A further investigation found a substantial association of the mutation rs2745150 in intron 11 of the CACNA1H gene with CAE. (Liang, Zhang et al. 2006). The CACNA1H mutation, (2025G>A) and (4867 G>T) in exons 9 and 10, was found in a family affect the CAE and FSs. This polymorphism is present in the splice-donor site of this gene, in exon 9b and 10a (Kananura et al. 2002). Whether these mutations affect the CACNA1H ion channel function or not, is unknown, but it was proposed that they may cause exon skipping, producing premature translation-termination codon (PTC), thus resulting in a non functional protein. This PTC might trigger nonsense-mediated decay (NMD). For that reason, it can be assumed that haploinsufficiency may be the underlying mechanism for this splice-donor site mutation. Position of (2025G>A) and (4867 G>T) in the exons 9 and 10 transition is the recognition sequence of AciI restriction enzyme. It is a type II restriction endonuclease from an *Arthrobacter citreus* source. Recognition sequence for AciI is a 4-base non-palindromic sequence 5’-CCGC-3’ where it cuts between the two cytosines, while on the opposite strand (5’-GCGG-3’) it cuts between the 5’-guanine and cytosine (Polisson and Morgan 1990). (2025G>A) and (4867 G>T) in the exons 9 and 10, these transition alter this recognition sequence and thus the presence of this mutation can also be verified by restriction digestion of this enzyme. The variability found in this study may be due to some other factors like heterogeneity present at genomic level and/or some clinical variables might also be involved that were not readily identifiable this time because the sample number of heterogeneous patients was very small. Hence, a much larger sample number will be required to accurately identify associated factors. Some electrophysiological experiments are also needed to revise the effects of this polymorphism and to analyze whether, after this
mutation in the subunit gene, the CACNA1H ion channel will express its normal function properly. In the last few decades, several mutations in many genes have been identified that were associated with epileptogenesis. This study has opened a new avenue in medical sciences in Pakistan, which will help scientists to work on genetic diseases following the methodologies used in this study. The outcome of this study can be further used to confirm the hypotheses through animal modeling and proteomics. This study has revealed several genetic factors involved in genetic diseases, which will help medical scientists for genetic counseling. The mutations found in this study added information to gene databanks, which ultimately helps scientists to develop gene therapies for genetic diseases. Epilepsy is a complex nervous disorder and it needs further study to explore the other gene mutations causing epilepsy in local population of Punjab and Pakistan that will ultimately help to develop genetic counseling strategies, gene therapies and prenatal diagnostic procedures for the population of Pakistan.

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Conflict of interest
The authors declare no conflict of interest

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