

BioScientific Review (BSR)

Volume 3 Issue 2, 2021

ISSN(P): 2663-4198 ISSN(E): 2663-4201

Journal DOI: <https://doi.org/10.32350/BSR>

Issue DOI: <https://doi.org/10.32350/BSR.0302>

Homepage: <https://journals.umt.edu.pk/index.php/BSR>

Journal QR Code:



Article:

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Article DOI:

<https://doi.org/10.32350/BSR.0302.05>

Article QR Code:



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Citation:

Irshad A, Sarwar N, Sadia H, et al. *In vitro* identification of CACNA1H in the 9th exon of Childhood Absence Epilepsy (CAE) patients using bioEdit and clustalW2. *BioSciRev*.2021;3(2):54–65.

[Crossref](#)



Estd. 1990

A publication of the
Department of Life Sciences, School of Science
University of Management and Technology, Lahore, Pakistan

***In vitro* Identification of CACNA1H in the 9th Exon of Childhood Absence Epilepsy (CAE) Patients Using BioEdit and ClustalW2**

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Article Info

Received: June 8th, 2021

Revised: August 4th, 2021

Accepted: August 31st, 2021

Keywords

DNA amplification, epilepsy, mutations, prenatal diagnostic, sequences

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. Multiple types of genetic mutations are involved in epilepsy. This study was aimed at investigating the coding regions of *CACNA1H* gene for analyzing the mutations involved in epilepsy. Blood samples of mutually unrelated true representatives of CAE were collected from the psychiatry department of various hospitals in Lahore. DNA was extracted using the standard protocol and the amplification of the *CACNA1H* region was achieved with specially designed primers. Later on, the analysis of the results was carried out via the sequencing of target fragments. Sequences were analyzed using the BioEdit software and then aligned with the help of the ClustalW2 software. A series of 12 unrelated patients with CAE were screened for mutations in the *CACNA1H* gene. No mutation was found in exon 9a. As 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 the 9th exon. Subsequent analysis of these fragments identified transition mutations (2025G>A) in exon 9. To conclude, there is a need to explore the site of the gene along with other gene mutations causing epilepsy in the local population of Punjab, Pakistan. It will help to develop genetic counseling strategies, gene therapies, and prenatal diagnostic procedures for the said population.

1. Introduction

Epilepsy is an ailment of the nervous system characterized by the onward development of seizures and by cognitive, psychological, neurobiological and social problems. Epileptic seizures are recurrent attacks which are short lasting, brief jerks of altered consciousness and motor activity [1]. According to the estimates of WHO, epilepsy affects 50-70 million people worldwide. It contributes to 0.75% of the global burden of disease and remains more common in underdeveloped countries as compared to the developed states [2]. In Asia, 23 million people suffer from epilepsy. The prevalence of epilepsy in Pakistan was reported to be 10.0/1000 [3, 4]. In some cases, epilepsy is inherited but in most cases, it is not. It is known from a combination of epidemiology and *in vitro* research that defects in a single gene or in a combination of genes, exacerbated by environmental factors, can lead to epilepsy [1]. Epileptic problems mainly arise in infants under the age of 8 months and are characterized by focal seizures. The clinical pathway is usually harmless with spontaneous resolution within 2 years and in most cases, normal development of the psychomotor functions occurs. There are various types of focal epilepsy such as benign neonatal family seizures (BFNS), benign family newborn infant seizures (BFNIS) or benign familial infantile epilepsy (BFIE) during the first year of life based on the age of development [5].

Epilepsy influences all age groups but for children, it entails a variety of issues that can have a bad impact on their childhood [6]. There are some types of idiopathic generalizations as well as some specific types of epilepsy which follow an autosomal dominant, monogenic (or mendelian) pattern of inheritance which increases the likelihood of the identification of the affected gene. These mutations are found in Ca, Cl, K, and Na

channels, adding to the list of channelopathies which influence excitable tissues [7]. 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 an increased current density. Many of the mutations are clustered around the intracellular linker connecting transmembrane segments S2 and S3 in the voltage sensor domain and affect the surface expression of the channel. While these mutations alone may not be sufficient to induce an epileptic phenotype, they can increase excitability in a manner that contributes towards the generation or propagation of seizures [8]. Molecular genetics has revolutionized the understanding of epilepsy, as genetic differences are responsible for the heritable variety among individuals, including disease susceptibility [9]. Most genes associated with childhood absence epilepsy (CAE) include ion channel genes, such as the calcium channel, GABA receptor, acetylcholine receptor and others. CAE is closely linked to the calcium channel genes CACNA1H and CACNG3 [10].

CACNA1H is the most important ion channel that acts on the ligand-gated calcium channels in the mammalian brain. The GABA_A receptor is trimeric and 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 [11].

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

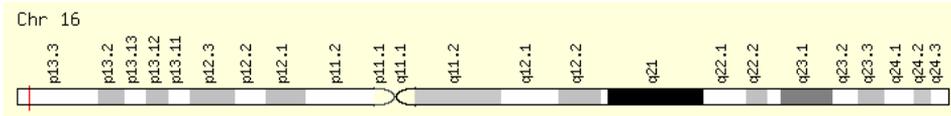


Figure 1. CACNA1H Gene Locus on Chromosome 16 [12]

Chromosome 16

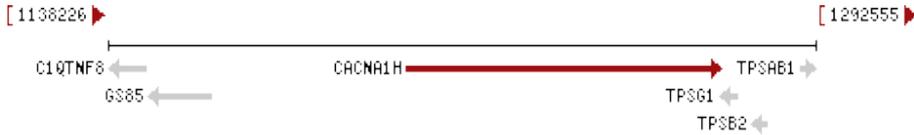


Figure 2. Map of CACNA1H Gene [12]

The current study was conducted to find out the mutations in the CACNA1H gene in CAE patients from Punjab, Pakistan. The purpose of this study was to evaluate the prevalence of CACNA1H gene mutations in the sensor region of exon 9a among CAE patients in Lahore with the help of literature review and the analysis of collected data.

2. Methodology

2.1. Selection of Individuals and Clinical Evaluation

First of all, individuals and families that have the neurological disorder CAE were identified. Patients were selected based on the clinical information provided by experienced neurologists. All available patients were interviewed and their seizure/epilepsy histories were documented by asking relevant questions about their individual relations, medical history. Further information was collected to exclude the onset of seizures due to environmental factors or any other disease which may cause seizures. Written informed consent was obtained from all the affected adults.

A total of 12 patients with CAE were identified from the pediatrics and the psychiatry departments of two different hospitals in Lahore (Sir Ganga Ram Hospital and Children Hospital Complex) and enrolled for the current study. All patients were less than 12 years of age. The

families of enrolled patients agreed to take part in the current study and allowed the researchers to collect blood samples (3-5 ml) of the affected individuals. A consent form was signed by the parents or the responsible adult. After DNA extraction, four primer sets were designed and synthesized to amplify the exons 9-11 of CACNA1H gene.

2.2. 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 at the Molecular Cytogenetic and Genomics Laboratory at the Institute of Biochemistry and Biotechnology, UVAS, Lahore. The samples were then stored (at -20⁰ C) for further processing.

2.3. DNA Extraction

The following materials and methods were used for DNA extraction and PCR amplification [13] with slight optimization and modifications.

2.4. DNA Quantification

After extraction, the quantification and analysis of DNA quality are mandatory to determine the approximate concentration of the DNA for further exploration. DNA

Table 1. CACNA1H Gene Primers

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

quantification was carried out using gel electrophoresis and spectrophotometric analysis.

2.5. Primer Designing

Primers were designed with the help of Primer3 software (<http://frodo.wi.mit.edu/primer3/>) 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.

Primers were synthesized as unlabelled from e-oligos (Table 1)

Primer sequences were optimized using the online tool OligoCalc (<http://www.basic.northwestern.edu/biotools/oligocalc.html>).

Their specificity was checked through the *in silico* PCR online tool available at the University of California, Santa Cruz (UCSC) genome browser (<http://genome.ucsc.edu/cgi-bin/hgPcr>)

2.6. PCR Amplification

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

2.7. Gel Electrophoresis

Amplification was confirmed using agarose gel electrophoresis. To check the

PCR product, 1.2% agarose gel was prepared using 1.2g of agarose. DNA ladder of 1 Kb was used as the molecular marker. Gel was viewed under UV light in the gel documentation system (Bio-Rad) and photographed. Then, PCR products was precipitated for sequencing.

2.8. Sequencing of PCR Products

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

2.9. 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 the manufacturer’s instructions given in the technical manuals. ABI PRISM sequencing analysis software (v. 3.7) was used to analyze the samples after each run.

2.10. Analysis of Sequencing Samples

The analysis of the sequences was carried out with the help of appropriate bioinformatics software and tools. Sequences were analyzed manually using the BioEdit software version (V.7.0). Nucleotide BLAST program, available at the NCBI website (<http://www.ncbi.nlm.nih.gov/BLAST>), was used for sequencing homology searches in public databases [14]. The sequences were also



BLAST against the reported reference sequence. Polymorphism was detected from the sequences through multiple sequence alignment using ClustalW2, an 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/>).

3. RESULTS

3.1. PCR Amplification of Exon 9

Figure 3 shows the PCR amplification of CACNA1H gene's exon 4. This exon was amplified using the CACNA1H ex9 primer set via 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 was used as molecular marker. The product size for this primer set was 450 bp.

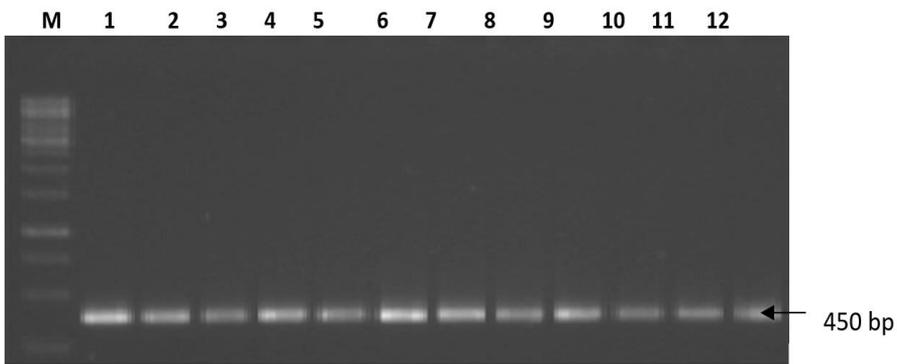


Figure 3. PCR amplification of exon 9

Lane M: Marker 1 kb SM0313 (Fermentas)

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

Product size: 450 bp

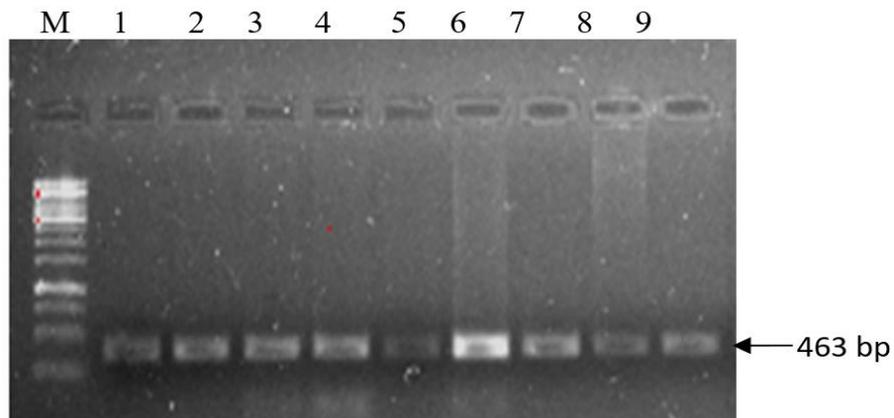


Figure 4. PCR amplification of exon 9

Lane M: Marker 1 kb SM0313 (Fermentas)

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

Product size: 463 bp

3.2. PCR Amplification of Exon 9

Figure 4 shows the PCR amplification of the exon 9 of the gene CACNA1H. This exonic region was amplified using the CACNA1H primer set following the touchdown PCR protocol. The annealing temperature range was 50-60°C. The product size of this primer (463 bp) was confirmed for the amplicons by gel electrophoresis.

3.3. 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 μ l. The purpose of precipitation was to eliminate the non-specific products from the amplified sample. These precipitated products were then sequenced for mutational analysis.

The current study was aimed at genetically characterizing the CACNA1H gene to identify mutations associated with CAE. Primers designed for amplification were based on the EPIC approach. Four amplification and sequencing primer sets were used for the analysis of mutations in exon 9. (Figure 5)

3.4. Mutation Found in Patients with CAE

A series of 12 unrelated patients with CAE were screened for mutations in the CACNA1H gene. No mutation was found in exon 9a. As previously reported, mutations in the exonic sequence of CACNA1H gene were found in 5 out of 4 CAE patients. These changes were observed in a PCR fragment amplified by primer 2 in the region of the 9th exon. Subsequent analysis of these fragments identified transition mutations (2025G>A) in exon 9 (Figure 6).

The schematic diagram above shows the location of the CACNA1H gene's exonic polymorphism. Red points show the hotspots involved in CAE. Green points depict the hotspots for IDE.

3.5. Results of SNP in Exon 9

The sense strand shows the normal sequence from blast. Subject strand has mutation at position 2025, where A is mutated by G (2025G>A) as highlighted in yellow colour (Figure 7) These mutations are in the sensor region of exon 9a giving the "gain in function" property to the on channels.(Figure 8a, 8b, 8c)

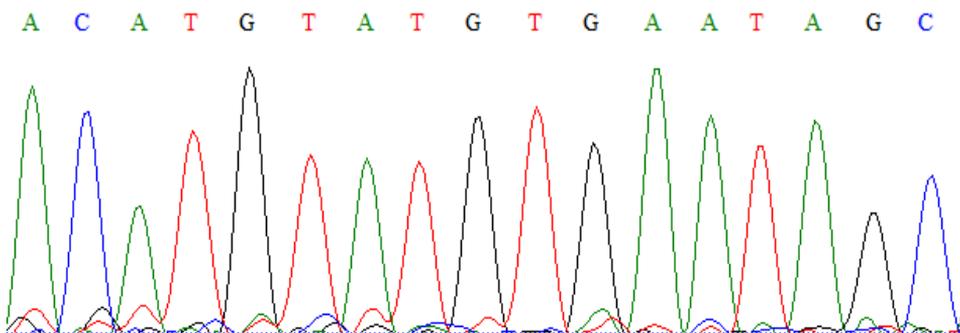


Figure 5. Chromatogram from exon 9a

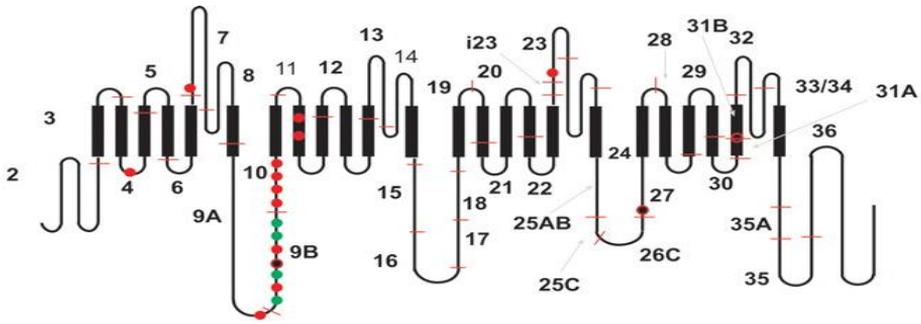


Figure 6. CACNA1H gene exonic polymorphism

T C T T T **G** T T A A G T A T G

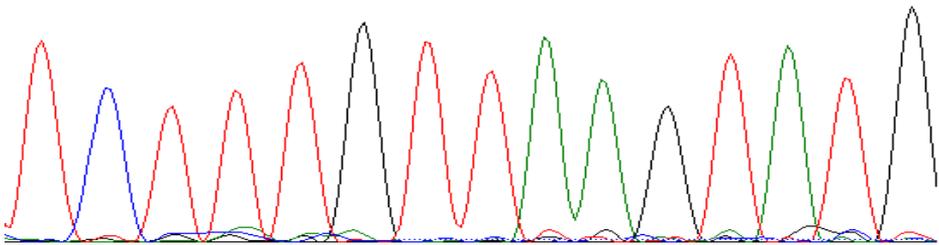


Figure 8 (a). Chromatogram from exon 9a (control)

T A T C T T T T T T A A G T A T G

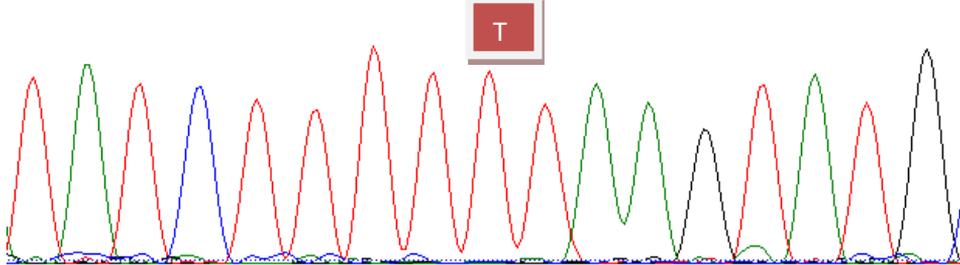


Figure 8(b). Chromatogram from exon 9b

A A T A T C T T T T T T A A G T

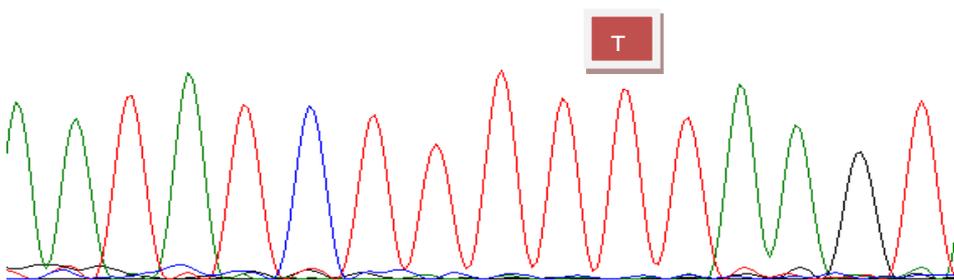


Figure 8 (c). Chromatogram from exon 9b

Query	86	TGTGAATTTACGTTGCCTTCACTGTAAGAGAAACATAGAAATGTGTGCACATAGAGAGG	145
Sbjct	243	TGTGAATTTACGTTGGCTTCACTGTAAGAGAAACATAGAAATGTGTGCACATAGAGAGG	184
Query	146	TGCAAGATTTTAAATGTTTTAATCTTTGTTTTCTAGAATAGATTTATTTAGCTACATTAGA	205
Sbjct	183	TGCAAGATTTTAAATGTTTTAATCTTTGTTTTCTAGAATAGATTTATTTAGCTACATTAGA	124
Query	206	AGTAATTTATTACAACATAATTTGGTACCAGTTACTAATTCACCACATTTGAATAACTAT	265
Sbjct	123	AGTAATTTATTACAACATAATTTGGTACCAGTTACTAATTCACCACATTTGAATAACTAT	64
Query	266	TTTGTTTTCAAAGAAATATCTTTTTAAGTATGCACATGCACCAAGCACGCATAGTAGAG	325
Sbjct	63	TTTGTTTTCAAAGAAATATCTTTTTAAGTATGCACATGCACCAAGCACGCATAGTAGAG	4
Query	326	TTT	
Sbjct	3	TTT 1	
Query	86	TGTGAATTTACGTTGCCTTCACTGTAAGAGAAACATAGAAATGTGTGCACATAGAGAGG	145
Sbjct	243	TGTGAATTTACGTTGGCTTCACTGTAAGAGAAACATAGAAATGTGTGCACATAGAGAGG	184
Query	146	TGCAAGATTTTAAATGTTTTAATCTTTGTTTTCTAGAATAGATTTATTTAGCTACATTAGA	205
Sbjct	183	TGCAAGATTTTAAATGTTTTAATCTTTGTTTTCTAGAATAGATTTATTTAGCTACATTAGA	124
Query	206	AGTAATTTATTACAACATAATTTGGTACCAGTTACTAATTCACCACATTTGAATAACTAT	265
Sbjct	123	AGTAATTTATTACAACATAATTTGGTACCAGTTACTAATTCACCACATTTGAATAACTAT	64
Query	266	TTTGTTTTCAAAGAAATATCTTTTTAAGTATGCACATGCACCAAGCACGCATAGTAGAG	325
Sbjct	63	TTTGTTTTCAAAGAAATATCTTTTTAAGTATGCACATGCACCAAGCACGCATAGTAGAG	4
Query	326	TTT	
Sbjct	3	TTT 1	
Query	76	TGTGAATTTACGTTGCCTTCACTGTAAGAGAAACATAGAAATGTGTGCACATAGAGAGG	125
Sbjct	143	TGTGAATTTACGTTGGCTTCACTGTAAGAGAAACATAGAAATGTGTGCACATAGAGAGG	164
Query	246	TGCAAGATTTTAAATGTTTTAATCTTTGTTTTCTAGAATAGATTTATTTAGCTACATTAGA	225
Sbjct	173	TGCAAGATTTTAAATGTTTTAATCTTTGTTTTCTAGAATAGATTTATTTAGCTACATTAGA	114
Query	106	AGTAATTTATTACAACATAATTTGGTACCAGTTACTAATTCACCACATTTGAATAACTAT	166
Sbjct		AGTAATTTATTACAACATAATTTGGTACCAGTTACTAATTCACCACATTTGAATAACTAT	74
Query	266	TTTGTTTTCAAAGAAATATCTTTTTAAGTATGCACATGCACCAAGCACGCATAGTAGAG	225
Sbjct	63	TTTGTTTTCAAAGAAATATCTTTTTAAGTATGCACATGCACCAAGCACGCATAGTAGAG	6
Query		TTT	
Sbjct	6	TTT 3	
Query	82	TGTGAATTTACGTTGCCTTCACTGTAAGAGAAACATAGAAATGTGTGCACATAGAGAGG	145
Sbjct	234	TGTGAATTTACGTTGGCTTCACTGTAAGAGAAACATAGAAATGTGTGCACATAGAGAGG	184
Query	164	TGCAAGATTTTAAATGTTTTAATCTTTGTTTTCTAGAATAGATTTATTTAGCTACATTAGA	205
Sbjct	138	TGCAAGATTTTAAATGTTTTAATCTTTGTTTTCTAGAATAGATTTATTTAGCTACATTAGA	124
Query	296	AGTAATTTATTACAACATAATTTGGTACCAGTTACTAATTCACCACATTTGAATAACTAT	265
Sbjct	132	AGTAATTTATTACAACATAATTTGGTACCAGTTACTAATTCACCACATTTGAATAACTAT	64
Query	289	TTTGTTTTCAAAGAAATATCTTTTTAAGTATGCACATGCACCAAGCACGCATAGTAGAG	325
Sbjct	78	TTTGTTTTCAAAGAAATATCTTTTTAAGTATGCACATGCACCAAGCACGCATAGTAGAG	4
Query	364	TTT	
Sbjct	7	TTT 1	

Figure 9. The sense strand shows the normal sequence from blast. Subject strand has mutation at position 2025, where A is mutated by G (2025G>A) as highlighted in yellow colour. These mutations are in the sensor region of exon 9a giving the “gain in function” property to the ion channels

4. Discussion

A set of primers with the product size of 450 bp and 463 bp were used for the amplification of CACNA1H gene's exon 4 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 based on the EPIC approach. There was no significant mutation found in exon 9a in the participants that were not the victim of CAE. Previously reported CAE patients' mutant genes were observed in a PCR fragment amplified by primer 2 in the region of the 9th exon. Subsequent analysis of these fragments identified transition mutations (2025G>A) in exon 9. 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 [15]. Sequences were analyzed through the BioEdit software and then aligned with the help of ClustalW2 software. It has been identified by a 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 [16].

In literature, many other such exonic and intronic mutations in CACNA1H receptor genes reportedly cause susceptibility to epilepsy. These mutations drop the CACNA1H expression level, resulting in gain in Ca²⁺ and gain of function of CACNA1H receptors by accelerated activation. CACNA1H polymorphisms (2025G>A) and (4867 G>T) in the exons

9 and 10 reportedly cause susceptibility to epilepsy and drug resistance [17]. The existence of 12 missense mutations in CACNA1H gene was determined by the mutational investigation of the Han Chinese population of CAE patients [18]. A further investigation found a substantial association of the mutation rs2745150 in intron 11 of the CACNA1H gene with CAE [11]. The CACNA1H mutations (2025G>A) and (4867 G>T) in exons 9 and 10 affect CAE and FSs. Polymorphism is present in the splice-donor site of this gene, in exon 9b and 10a [19]. Whether these mutations affect the CACNA1H ion channel function or not is unknown, although it was proposed that they may cause exon skipping while producing premature translation-termination codon (PTC), thus resulting in a non-functional protein. The PTC might trigger nonsense-mediated decay (NMD). For this reason, it can be assumed that haploinsufficiency may be the underlying mechanism for this splice-donor site mutation. The positions (2025G>A) and (4867 G>T) in the exons 9 and 10 transition comprise the recognition sequence of AclI restriction enzyme. It is a type II restriction endonuclease from an *Arthrobacter citreus* source. The recognition sequence for AclI 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 [20]. As far as the positions (2025G>A) and (4867 G>T) in the exons 9 and 10 are concerned, these transitions alter this recognition sequence and thus the presence of this mutation can also be verified by the restriction digestion of this enzyme. The variability found in this study may be due to factors such as the heterogeneity present at the genomic level. Moreover, some clinical variables might also be involved that are not readily identifiable because the sample number of heterogeneous patients was very small.

Hence, a much larger sample number is required to accurately identify the 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 expresses its normal function properly. In the last few decades, several mutations in many genes were identified that are associated with epileptogenesis. This study has opened a new avenue in medical sciences in Pakistan which will help the scientists to work on genetic diseases following the methodology used in this study. The outcome of this study can be further confirmed using animal modeling and proteomics. This study revealed several genetic factors involved in genetic diseases which will help medical scientists in genetic counseling. The mutations found in this study add information to the gene databanks, which will help the scientists to develop gene therapies for genetic diseases.

Acknowledgement

The authors are very grateful to the Higher Education Commission (HEC), Pakistan and all the researchers for their support.

Conflict of interest

The authors declare no conflict of interest.

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