

MOLECULAR ANALYSIS OF LGI4 GENE MUTATION IN JUVENILE MYOCLONIC EPILEPSY PATIENTS IN DRAVIDIAN LINGUISTIC POPULATION IN SOUTH INDIA

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ABSTRACT: Juvenile Myoclonic Epilepsy (JME) or Janz syndrome is inherited disorder, otherwise neurologically normal.⁽¹⁾ The prevalence of JME is estimated around 3 in 10, 000. The genetic mutations in JME patients affecting non-ion channels and the exact mode of JME inheritance are not clear. Mutation within an exon of a structural gene can alter the functioning of the gene product and cause a dramatic phenotypic change. **METHOD:** The case-control association study design was used to test the potential involvement of LGI4 gene variations in the etiology of JME. We analyzed mutation for molecular screening of LGI4 sequence coding 2-3 exon. Detection of mutation was performed by genomic PCR amplification and direct sequencing by ABI PRISM® 377 DNA Analyzer. **RESULT:** We identified novel mutation (36%) in LGI4 gene changes G-to-N transversion at base pair 112 polymorphic site in exon 2-3. The novel mutation can change to the alteration of a chromosomal loci on 19q 13.11. The LGI4 gene secreted glycosylated leucine-rich repeat protein that involved in Schwann cells for the formation of myelin sheath. Demyelination of sheath may damage the axon which leads to JME. **CONCLUSION:** The exact mechanism of JME involved different gene mutations is still unknown. Further Familial and twin studies are required to investigate the strong involvement of LGI1, LGI2 and LGI4 genes in the genetic susceptibility of JME syndrome for the role of CNS and PNS.

KEYWORDS: JME, LGI4 gene, nonsense mutation, myelin sheath, PNS.

INTRODUCTION: Juvenile Myoclonic Epilepsy (JME) was first described by Janz and Christian in 1957. JME is a genetically determined an idiopathic generalized epilepsy (IGE) syndrome and probably involves multiple genes due to widespread distribution in the central nervous system (CNS) and minor influence of environmental factors were contributed. The genetic background for JME account for 5-10% of all form of epilepsy. The age of onset is between 8 to 18 years with peak age at 14.5 to 15.5 years that affects both genders. The prevalence of JME: 3 per 10, 000.^[2] (Radhakrishnan; 2000).

The identification of these susceptibility genes are great challenge in JME patients and we approach to identify Leucine-Rich Glioma Inactivated Protein 4 (LGI4) gene based on the genetic molecular analysis studies involved in non-ion channel gene. The symptoms with JME is caused by multiple genes that are associated with as cortical development, mitochondrial function and cell metabolism, each 'epilepsy gene' that is identified new and fascinating insight into the molecular basis of neuronal excitability and brain function. "The activation of certain neural

ORIGINAL ARTICLE

regions and mutations believed to disrupt the massive neural discharge that leads to epileptic seizures".⁽³⁾

Type of epilepsy	Chromosome	Gene linkage	Gene products
JME	19q13.11	LGI4	Leucine-rich glioma inactivated 4 protein Total gene size: 10762 bp

Table 1: Gene identified in inheritance of JME syndrome in the present study

Anatomy in relation with LGI4 gene: All axons in the PNS are surrounded by Schwann cells and undergo a wrapping process called myelination. Schwann cells cover the axons with many layers of plasma membranes, containing lipids and proteins called myelin sheath. The myelin sheath act as an insulator along with node of Ranvier and conduction velocity in the myelinated axons (12-20 um in diameter) is 70-120 m/sec at the node of Ranvier, and voltage-gated sodium channels are highly accumulated and generate action potentials. Schwann cell is about 0.3 mm to 1.5 mm in length and contains outer membranous called neurolemma. The damage or loss of myelin sheath leads to various neurological symptoms. In CNS, myelin sheath is produced by Oligodendrocytes and PNS produced by Schwann cells. During the development of PNS myelinated nerve fibers a molecule called gliomedin is secreted from myelinated Schwann cells. The LGI4 gene secreted glycosylated protein involved in Schwann cell growth. The genetic mutation that affects the glial cells, which damage the myelin sheath and process is called demyelination.

LGI4 gene: LGI4 gene found in Schwann cell of PNS and the role of Lgi4 in CNS is limited, CNS myelination as epilepsy may involve other genes, because JME disorder inherited by the multiple genes.

Leucine-rich Glioma-Inactivated protein 4 (LGI4) also known as leucine-rich repeat LGI family member 4 which contains 537 amino acid and secreted glycosylated protein that is widely expressed, with highest levels found within the peripheral nervous system.⁽⁵⁾ Lgi4 which belongs to super family of leucine-rich repeat (LRR) protein. The sequence variations in LGI4 in combination with other genetic factors might modulate this developmental and functional process leading to the expression of JME symptoms. The myelin sheath surrounds a large fraction of nerve fibers in the vertebral nervous system. The myelinated nerve fibers faster than unmyelinated fibers and myelin sheath play key role for normal function of nervous system and dysfunction of sheath associated with neurological disease.

MATERIAL AND METHOD: The present study was carried out in the Department of Anatomy, Krishna Institute of Medical Sciences University, Karad (M.H) through Sandor Proteomics Pvt Ltd, Banajara Hills, Hyderabad.

MATERIAL:

Human subjects: Unrelated JME probands were recruitment from three regions of Kosta Andhra, Rayalaseema and Telangana of Andhra Pradesh (AP) in South India. All JME patients

were diagnosed according to the guidelines based on the Commission on Classification of Epilepsies and Epileptic Syndromes of the International League against Epilepsy (Berg et al, 2010).

A total unrelated 75 JME patients of Male-44, and Female-31 mean age male-8.11 and female-9.4 were recruited for the study. We analyzed 100 healthy control individuals (60 male and 40 female) of the same age and gender matched with similar ethnicity and had no evidence of family history of neurological disorder of epilepsy. The participation rate was 100% in the present study.

Ethics approval: Molecular genetic analysis on human experiment of study protocols was approved by the Ethical committee of the faculty of Medicine on Human Research by Krishna Institute of Medical Science Deemed University, Karad, and written informed consent was obtained from all JME patients and control subjects. The primary diagnosis of JME patients had based on clinical criteria and laboratory findings.

DNA extraction: A volume of 5 ml of venous blood were collected from all participants in EDTA (Ethylene diamine tetra acetic acid) vacutainers tube and stored at -70°C until further use. Genomic DNA (nuclear and mitochondrial) was extracted from peripheral blood leukocytes by using the phenol-chloroform method. The extracted DNA was quantified by the spectrophotometer method followed by checking in 2% agarose gel and DNA samples were stored at -20°C .

METHODS:

Nuclear DNA:

Oligonucleotide Amplification and sequence Analysis of LGI4 using specific primers:

The Sanger method for DNA sequencing was first described over 30 years ago (sanger et al., 1997). The LGI4 is amplified by using nine primers of LGI4 gene. The primers used in this study designed by Eurofins genomics, Bangalore.

The PCR reaction mixture (total volume, 10 μl) containing 50 ng genomic DNA, 2.5 μl of 10x PCR reaction buffer, 1.5 mM MgCl_2 , 1.0 μl of 25 mM of deoxyribonucleotide triphosphate (dNTP), primers forward and reverse at 5 pmol/ μl , digestion of the PCR product was done with 0.4 μl of Taq enzyme (TAKARA, India)) in the reaction buffer and 7.9 μl of sterile water for dilution.

All the samples were amplified in the following conditions: 7 min denaturing step at 35 cycles, each cycle: denaturation 95°C for 30 sec, annealing $55-62^{\circ}\text{C}$ for 45 sec, elongation 72°C – 1.3 min, repeated for 35 cycles followed by a final extension step at 72°C for 7 min. (primer sequences were obtained from this study carried out by Gillis et al 2004).

The amplified PCR products were separated by electrophoresis using 2% agarose gel run at 110V for 15 min from positive charge to negative charge. The PCR product of gel plate placed in alpha imager through UV rays and the image was visualized on monitor for analysis of the quality of the primers.

ORIGINAL ARTICLE

LGI4 gene DNA analysis: DNA sequence using ABI 3730 DNA analyzer and sequencing of LGI4 carried out in Forward and Reverse direction for at least three times. The base change position of LGI4 was identified from sequenced data using bioinformatics tools. Amplifications size were verified by gel electrophoresis by running the PCR product on 2% agarose gel with the 100 bp marker, after successful amplifications, PCR products. PCR products were purified and subject to cycle sequencing using the Big DyeTM Terminator v 3.1 kit and sequenced by ABI PRISM® 377 DNA Analyzer for DNA analysis.

Figure 1: shows primer sequences, annealing temperature (56_C and 58_C) along with product size of LGI4 gene. Agarose gel picture showing LE-LGI4, exon1 to 9.

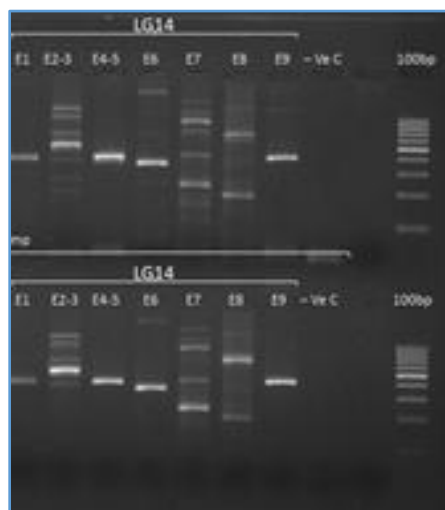


Figure 1

Primers/exons	Primer sequence		Amplicon size (bp)	Annealing temperature (C*)
	Forward	Reverse		
LG14/E1	5'GTGGAGGAGGAGACGTAG T3'	5'TCTCCTGAGATCCTGATG3'	364 bp	58.8and 53.7
LG14/E2-3	5'TGGTGTCCCTCTCACCTAC C3'	5'AGACCCACCTGCCTCCAT3'	318 bp	61.4 and 58.2
LG14/E4-5	5'CCA GTGGGCCTATGTCTTTC3'	5'GACACAAACATTCCCCAACC3'	383 bp	59.4 and 57.3
LG14/E6	5'CATCTCTGGGGACACTTG3'	5'AGTGAGACGAGCCTCTTG3'	341 bp	56 and 56
LG14/E7	5'GCCTGCCTGACTCTTTCT C3'	5'GCTGGACAGGCAGAAGGAC3'	228 bp	61.4 and 61
LG14/E8	5'CGTCCTTCTGCCTGTCCA3'	5'GGCATGTGAGGGTAGGTCAG3'	662 bp	58.2 and 61.4
LG14/E9	5'GCTTCTGCTTCCAAGCTGT T3'	5'CAGAGTCCCGTTGGTGGT3'	410 bp	57.3 and 58.2

Table 2: LGI4 Exon primer sequences, annealing temperature and size of the product

ORIGINAL ARTICLE

RESULT AND DISCUSSION: In our case-control study of 75 JME patients in which, 44 were male and 31female. The average age at onset of epilepsy was 14.24 years (range 7–20 years). Fourteen subjects (18.6%) had a positive family history of epilepsy. The 61 JME (81.3%) patients met the criteria for classic JME.

Sequencing analysis of LGI4 gene: Normal and aberrant single-strand conformational polymorphism (SSCP) conformers from each exons from 1-9 in LGI4 genes in five JME patients and ten healthy control subjects of the same age group were excised directly from 2% agarose gel and sequenced according to the ABI Big Dye Terminator Cycle sequencing protocol and using ABI 377 Automated sequences instrument.

The complete coding sequence of Lgi4 is amplified from genomic DNA and using Reverse and Forward primers at 4_ C and room temperature following standard protocols.

The following samples were used for Lgi4 gene

Lgi4 JME samples SP 5285, SP4995, SP4996, SP4997 and SP5075

Figure 2: PCR amplification with nine exons in LGI4 genes using Forward Primer (FP) and Reverse Primer (RP) with agarose gel 2% stained with ethidium bromide for visualizing DNA bands of the fragment migration:

Amplification and sequencing of LGI4 patients and normal subject control.

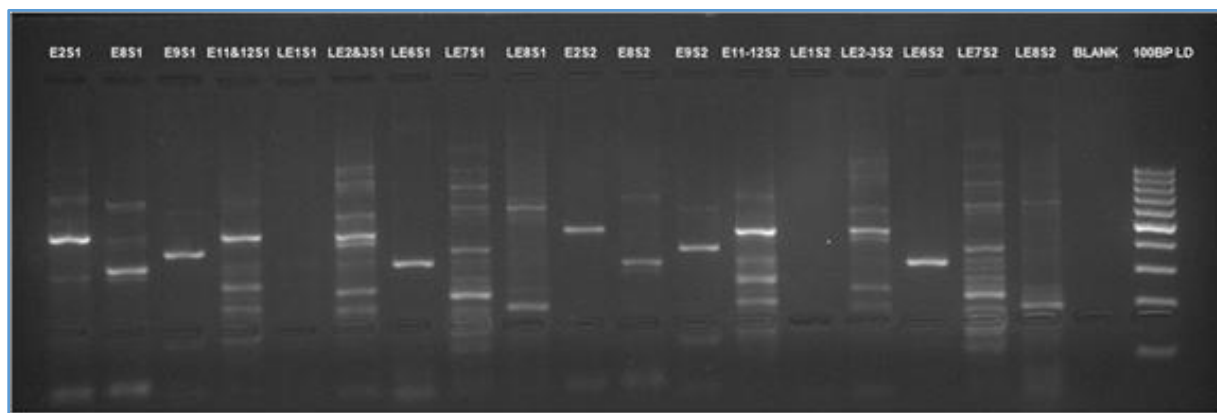


Figure 2

BLAST search was used to identify homology between the sequence obtained from the case test (CT) are evaluate sequence of case control (CC).

We follow PCR amplification for DNA sequence in 70 JME patients with exon 2-3.

Location and Exon	Primer sequence	Annealing temperature	Length (bp)
LGI4- 2-3	(F) 5'TGGTGTCCCTCTCACCTACC3' (R) 5'AGACCCACCTGCCTCCAT3'	61.4 and 58.2	318 bp

Table 3: Oligonucleotide amplification primers and PCR condition

ORIGINAL ARTICLE

Mutations detected and identification of SNPs

Polymorphisms in LGI4: We identified one autosomal recessive type of novel mutation changes G-to-N (Glycine-to-Asparagine) transversion at base pair 112 in exon 2-3 in LGI4 gene. These mutations are not been previously reported in the single nucleotide polymorphism NCBI database (SNPs). The first identified mutation, observed in sample 4996-4997 G/n, in LGI4 gene.

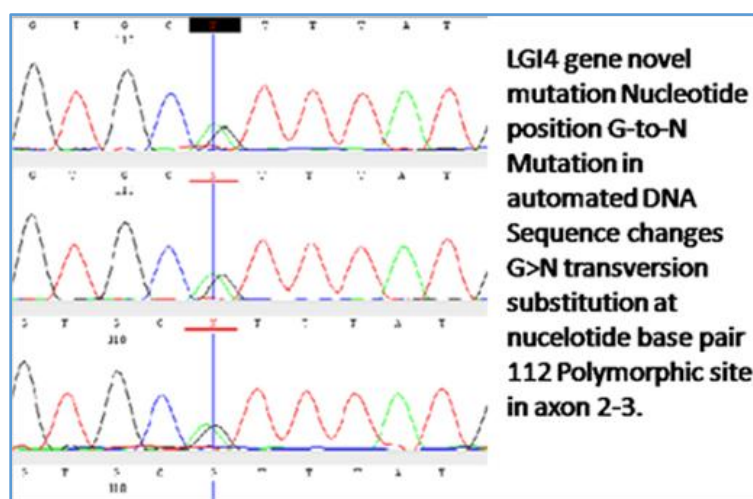


Figure 3

Genotype (G)	Allele (A)	Control (n=100)		Cases (JME) (n =75)		Chi: square	P value
		G	A	G	A		
CGTT	CNTT	100	-	48 (64%)	27 (36%)	39.853	0.000
GAGT	GAAT	96	04	75	-	1.540	0.215
CACA	CCCA	89	11	71 (94.6)	04 (5.3)	1.107	0.293

Table 4: Genotype and Allele frequency distribution of LGI4 SNPs in JME case-controls

Allele G/N is shows highly significant and P value is = 0.000

DISCUSSION: LGI4 gene contains 537 amino acid and secreted glycosylated leucine rich protein that involved in Schwann cells. The Schwann cells act as signaling pathways, that controls axon segregation and myelin formation.⁽⁶⁾

The structural and functional mutation of myelinated nerve fibers in the peripheral nervous system (PNS) depends on the molecular interaction between the axon and Schwann cell (Jessen and Mirsky, 2005).

LGI4 gene mutation has been shown to underlie the congenital hypomyelinating phenotype of claw Paw mice (Bermingham et al. 2006) in PNS.

LGI4 protein is implicated as a positive regulator of myelin formation in the PNS and polymorphism in Lgi4 gene may reduce myelin formation in neuron-Schwann cell and causes hypomyelination in the peripheral nerve (Bermingham et al. 2006).

ORIGINAL ARTICLE

LGI4 gene is expressed by neural crest stem cells but its expression restricted to the glial cells, which exist throughout the nervous system and include Schwann cells, astrocytes and oligodendrocytes.⁽⁷⁾ The neuronal axons and dendrites become coated with a segmented lipid-rich sheath (myelin) to enable faster and more energetically efficient conduction of electrical impulses. The myelin sheath is formed by the cell membranes of Schwann cells in PNS.⁽⁵⁾ The presence of an altered single amino acid change in a LGI4 protein reflects a point mutation. LGI4 gene by contrast, a mutation within an axon of a structural gene can alter the functions of the gene product and cause a phenotypic change. The mutations in the DNA germ line cells may be transmitted by gametes to the next generation.^(3, 4)

A nonsense mutation is N-terminus, may change to a single base pair to the alteration of a large region of chromosome loci on 19q13.11. A nonsense mutation occurs when a nucleotide substitution changes a codon that specifies an amino acid into one that is a stop codon. Patients with juvenile myoclonic epilepsy showed increased functional connectivity between the motor system and frontoparietal cognitive networks.

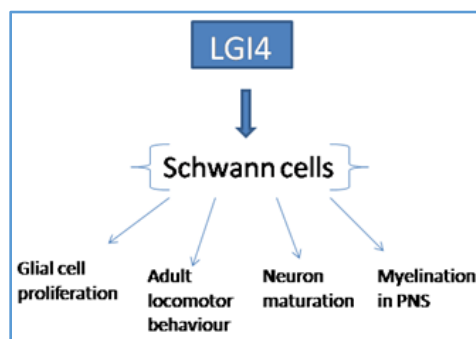


Fig. 4: Scheme to show the function of LGI4 gene in Schwann cells

The location of a gene at chromosome 6p21.3 and 19q13.11 are confined by several independent studies of the common form of JME (Greenberg et, al. 1988b, Durner et, al. 1991; Weissbecket et, al. 1991; Sander et. Al. 1997).

CONCLUSION: Non-ion channel genes (Lgi4) were also contribute the Myelin sheath increase speed of electrical impulses approximately two orders of magnitude faster than unmyelinated fibers of similar diameter and gives insulates the axon and assembles voltage-gated sodium channel cluster at discrete nodes. Demyelination of sheath underscored by the large range of neurological diseases associated with its dysfunction. However, more studies are required to add to the pool of information and to help us to better understand the genetic structures of diverse in South Indian population groups, where many questions remain unanswered. The exact mechanism of JME involved different gene mutations is still unknown. Further Familial and twin studies are required to investigate the strong involvement of LGI1, LGI2 and LGI4 genes in the genetic susceptibility of JME syndrome and role in CNS and PNS.

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