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Human Genetics

Point Nonsense Mutations in BRD2 gene of Juvenile Myoclonic Epilepsy (JME) patients: A study from Dravidian Linguistic South Indian Population.

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ABSTRACT

BACKGROUND : Juvenile myoclonic epilepsies (JME) are primarily genetic in origin and genetically determined adolescent syndrome among the idiopathic generalized epilepsies (IGE) probably involves multiple genes. Direct sequencing of the BRD2 gene revealed a heterozygous point nonsense mutation (C>T and A>T) in JME patients. Molecular genetic analysis revealed that both were heterozygous for a nonsense mutation in Brodamine containing protein-2 (BRD2) in exon 9 which lead to a definite diagnosis of JME. The genetic background for JME accounts for 5-10% of all form of epilepsy. A non-ion channel gene of the BRD2 protein cause programmed cell death in the developing brain. The JME is estimated around 3 in 10,000 with peak age at 14.5 to 15.5 years that affects both genders. Mutation within exons of a structural gene can alter the functioning of the gene product and cause a dramatic phenotypic change in amino acid sequence.

OBJECTIVES

1. To support innovative research, of the highest scientific merit, that has the greatest potential for patient benefit.
2. To identify the mutation of BRD2 gene in nuclear DNA in JME patients

METHODS : The case-control association study design was utilized to test the potential involvement of BRD2 gene variations in the etiology of JME. We performed molecular screening of BRD2 gene coding sequence for the detection of mutation through genomic PCR amplification and direct sequencing by ABI PRISM® 377 DNA Analyzer.

RESULT: These nonsense mutations involved exon 9 and most of them are autosomal dominant.

Direct sequencing of the BRD2 gene showed a heterozygous nonsense mutation at nucleotides 254 and 896 base pairs of exon 9 in two affected unrelated JME cases.

INTERPRETATION & CONCLUSION: The exact genetic mechanism of JME is still unknown due its multiple genes involvement. Further Familial and twin studies are required to investigate the involvement of BRD1, BRD2 and BRD3 genes in the genetic susceptibility of JME syndrome in central nervous system.

KEYWORDS : BRD2 gene, JME, M.J, Nonsense mutation, SNP, WES

INTRODUCTION

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 back ground for JME account for 5-10% of all forms of epilepsy. About 7% of the siblings of probands with JME and in all type of epilepsy of which 30% will have JME **(1)**.The age of onset is between 8 to 18 years with peak age at 13.5 to 15.5 years that affects both genders. The prevalence of JME: 3 per 10,000 (Radhakrishnan; 2000).

The identification of susceptibility genes are great challenge in JME patients and we approached to identity the genetically associated with Bromodomain containing 2 protein (BRD2) gene through molecular analysis. JME has been linked to mutations in CACNB4, EFHC1, GABRA1, CSTB, EPM2A, EPM2B, and PRICKLE1.**(2,3)**. The BRD2 gene located on chromosome 6p21.3 (human leukocyte antigen [HLA] region) and associated with HLA alleles **(4)**. All two generations of family (patient and sibling) showed a autosomal dominant inheritance pattern of the disease, and genetic molecular studies confirmed that JME patients had nonsense mutations observed in 75 JME samples; however, the 100 normal controls did not show similar changes. Variant in BRD2 gene related with disorganized neuronal connectivity and neurocortical-hyperexcitability and SNPs in BRD2 (RING3) gene is susceptibility of autosomal recessive JME in many cases **(5)**. BRD2 spans approximately 399 BP of DNA, and consists of 12 exons and 11 introns. It encodes the protein product, and consisting of 88061 amino acids. The defects in genes coding for ion channel proteins is one of the genetic cause of idiopathic forms of epilepsy **[6]**. The age of onset is usually begins from puberty (late childhood), affects both the genders equally and is characterized by myoclonic jerks, frequently generalized tonic-clonic seizures, and some times absence seizures.**[7,8]**. Interictal and ictal EEG is characterized by generalized spike-wave, multiple spikes and slow-wave discharges with normal background activity.**[9].** The major clinical characteristics of JME include myoclonic jerks and generalized tonic-clonic seizures. Some patients also suffer from absence seizures. JME is considered a benign form of epilepsy with a good prognosis when treated with antiepileptic drugs **[10].** The clinical symptoms of JME may related to multiple genes that are associated with cortical development, mitochondrial function and cell metabolism, Each 'epilepsy gene' is contributes to the understanding fascinating insight into the molecular basis of neuronal excitability and brain function. "The activation of certain neural regions and mutations believed to disrupt the massive neural discharge that leads to epileptic seizures. The role for BRD2–a transcriptional regulates brain development and errors in regulation might explain the basis of development of JME.

Material and Method

Recruitment of patients

The 75 unrelated JME patients in which 44 males and 31 females was recruited from Ethnic Dravidian population of South Indian three states of Andhra Pradesh (AP) and Telengana (T.S) and Karnataka (Hyderabad-Karnataka region). The present study was carried out

(2001-2014) in the Department of Anatomy, Krishna Institute of Medical Sciences University, Karad (M.H) in collaboration with Sandor Proteomics Pvt Ltd, Banajara Hills, Hyderabad (T.S). The primary diagnosis of JME patients was based on EEG, CT/MRI and clinical findings under the supervision of consultant Neurologist with special interest in epilepsy. All JME patients were selected based on the diagnostic evaluation made according to the classification of the International League Against Epilepsy (ILAE) **[11]**.

Interview and sample collection

The JME patients were interviewed in person with a standard questionnaire under the direction of epilepsy specialist. The questionnaire gathered their past medical histories, seizure patterns and their characteristic, EEG pattern, states of seizure control, medical investigations and their medications in controlling epilepsy. During the interview draw the 5 ml of peripheral blood was collected and transferred into EDTA tubes. The sample was also collected through Website, print media and pamphlet. The sample was included ♂ and ♀

Normal Healthy Control

To assess the possible occurrence of polymorphisms in any detected nucleotide substitutions, blood samples were also obtained from a total 100 normal healthy subjects (60 males and 40 females) from late childhood, adolescence and adults with the same ethnic background from the three states of Andhra Pradesh, Telangana and Karnataka (H-K region) states of South India. The controls had no history of neurological disease or family history of epilepsy and had normal structural neuron-imaging.

Ethical 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, M.H, India. Written informed consent was obtained from all the participants.

DNA isolation

A volume of 5 ml of venous blood were collected from all JME and control subjects and transferred into EDTA (Ehylene diamine tetra acetic acid) vacationers. These EDTA tubes were brought back to the Sandor Protoemics Laboratory, Banjarahills, Hyderabad (TS). Genomic DNA (nuclear and mitochondrial) was extracted from peripheral blood of leukocytes by using the phenol-chloroform method (Sambrook et al.1989). The extracted DNA was quantified by the spectrophotometer method followed by checking in 2 % agarose gel (Maniatis *et, al.,* 1989) and was stored at -20°C. The genomic DNA was precipitated in 100% ethanol and was then removed into a tube containing 0.5 ml of sterile 1xTE. The extracted DNA samples were stored in freezers at -72°C until further use for molecular analysis.

METHODS

PCR Amplification of Nuclear DNA

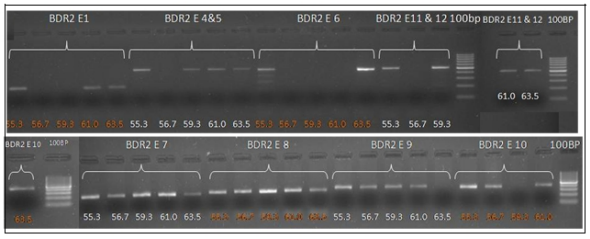
The BRD2 is amplified by using 10 primers for 12 exons of the gene. The primers were used in this study designed by Eurofins genomics, Bangalore.

Table:1 Description of the oligonucleotides used for the analysis of the BRD2 gene

Primers/exons	Primer sequence		Amplicon size (bp)	Annealing temperature (C*)
	Forward	Reverse		
BRD2/E1	5'CTTAGCGGGTTATGCTGGAC3'	5'CCGCTCAGTACTCCCAACAC3'	209 bp	59.4 and 61.4
BRD2/E2	5'TAAGCTTAACCACCTCACTAGG3'	5'CATCTACACTAGGCAGACCAC3'	496 bp	58.4 and 59.8
BRD2/E3	5'GGATCGGTAGTCTCCCTATAA3'	5'CTACCTGGATAACACCTTCAGT3'	321 bp	57.9 and 58.4
BRD2/E4-5	5'TCTTTATTGCTGTCTGTGTTCTCA3'	5'CCCAGAGGAAATCCACAGAT3'	487 bp	57.6 and 57.3
BRD2/E6	5'AAGTGGGCTTGGAGTGACAG3'	5'CACCTAGGCTCCCATCACTG3'	475 bp	59.4 and 61.4
BRD2/E7	5'GCTCTTCTTGTGGTGTCT3'	5'CTGTACAGAACAGTGAGACC3'	312 bp	53.7 and 57.3
BRD2/E8	5'GCTGGGTATGTAGGGCACTG3'	5'CCCAATAAAAACTTTCAAGAGTGA3'	338 bp	61.4 and 55.9
BRD2/E9	5'CATGCCCTTTGTCCTCATTT3'	5'CATCCCCCAGAGAGACAGAA3'	399 bp	55.3 and 59.4
BRD2/E10	5'TTTTGCTGACAACTCTTTTTGC3'	5'AGACCCCACCATCTTTCCTC3'	396 bp	54.7 and 59.4
BRD2/E11-12	5'GGGGCCCATAATAAGATGCT3'	5'GTCTAGGGGTCCGGTCCTG3'	489 bp	57.3 and 63.1

The PCR reaction mixture (total volume, 10 µl) containing 50 ng genomic DNA, 2.5 µl of 10x PCR reaction buffer, 1.5 mM MgCl2, 1.0 µl of 25 mM of deoxyribonuceotide 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 applying following conditions: 7 min denaturing step at 35 cycles, each cycle: denaturation 95°C for 30 sec, annealing 55-62°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 analyzed on 2% agarose gels electrophoresis, stained with ethidium bromide and visualized with UV light. Sequencing was performed in the Sandor Proteiomics Laboratory, Banjara Hills, Hyderabad (T.S) due to non availability of molecular genetics laboratory in the Department of Anatomy, Krishna Institute of Medical Sciences, Karad. M.H.

Fig:1 Gel picture showing the amplification produces of 12 exons of 3 JME patients



Epidemiology

JME patients begin the seizures between ages 12 and 18 with a mean age at onset 14.6 years, with range from 9 to 40 years. The mean age of onset for GTCS is 15.5 years, absence seizures 11.5 years, and myoclonic seizures 15.4 years.[12]

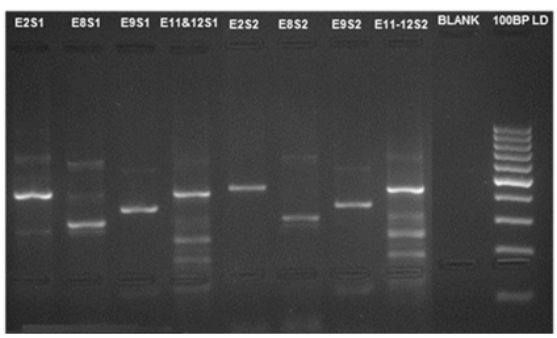
RESULT

DNA sequencing analysis of BRD2 gene

The complete coding sequence of BRD2 gene is amplified from genomic DNA using standard protocols. We randomly selected three samples in out of 75 JME patients and the entire 12 exons were sequenced initially. Based on the result of molecular analysis in three samples, we found nonsense mutations in exon 9 and decided to process the remaining 72 JME samples and the 100 normal control samples for sequencing to the exon 9 only for identify the mutations.

The sequencing analysis of BRD2 genes was screened by double strand conformational analysis (DSCA) by using ABI 377 automated sequences instrument. Sequenced according to the ABI Big Dye Terminator Cycle sequencing protocol. JME dominated among siblings and phenotypic heterogeneity was observed in second and third degree relatives. BIAST search was used to identify homology between the sequence obtained from the case test are evaluate sequence of case control.

Figure:2 Representation of DNA sequencing electrophreogram analysis of BRD2 gene



Mutation analysis

We selected initially three JME patients and five unaffected (free from JME) control subjects of the same gender for direct sequencing of all 12 exons in the BRD2 gene. The results revealed a heterozygous nonsense mutations observed only in exon 9. However these mutations

are not found in the control subjects. We further sequencing on 72 JME patients in particularly exon 9 for detecting the mutations, which may result in two nonsense mutations seen in two males in BRD2 gene. A nonsense mutation changes a triplet codon that encodes a specific amino acid or stop codon. The DNA sequence shortened the protein products of BRD2 gene due to stop codons in this phenomenon. The nonsense mutation may leads to protein is incomplete, and shows nonfunctional proteins.

Table: 2 Nonsense mutations associated with BRD2 gene in JME cases and seizures type

Base pair	Allelic variants	Position of Exon	Subtype of mutation	Aminoacid changes	Age at onset	Type of seizure	Gender
Bp 254	CAG > TAG	Exon-9	Nonsense	Glu > Stop	15	Myoclonic	M
Bp896	AGA > TGA	Exon-9	Nonsense	Arg > Stop	27	GTCS	M

The codon for one amino acid is replaced by a translation-termination (Stop) codon in nonsense mutation. The first pathogenic allele was identified in whole-exone sequencing (WES) has revealed a base substitution of sequencing analysis reveling a C > T transition resulting amino acid substitution and protein terminates at this point. We found in two nonsense mutations in exon 9 in 75 JME samples: SP-5075, SP-5223 changes of nucleotides at 254, 896 base pairs. A nonsense mutation is also a change in one DNA base pair and instead of substituting one amino acid for another; however, the altered DNA sequence prematurely signals the cell to stop building a protein.

There is no such mutations were found in the 100 normal healthy subjects. These mutations have not been previously reported in the single nucleotide polymorphism NCBI database (SNPs). Variations in the amino acid sequence of certain proteins can cause JME disease. The case-control study suggests that BRD2 gene is plying a major contributor to JME patients. The SNPs observed in BRD2 gene identified here will be useful in future association and linkage studies.

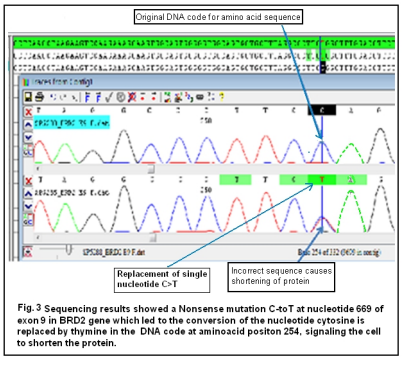


Fig. 3 Sequencing results showed a Nonsense mutation C-toT at nucleotide 669 of exon 9 in BRD2 gene which led to the conversion of the nucleotide cytosine is replaced by thymine in the DNA code at aminoacid positon 254, signaling the cell to shorten the protein.

DISCUSSION

During sleep, specialized neurons in the thalamus with profuse connections to the entire brain gradually disrupt the individual activities of cortical neurons and entrain them all into monotonous rhythmic synchronized discharges **(13)**. Therefore, synchronized activity of large numbers of neurons abolishes their normal 'wakeful' functions.

In recent report of neuron-anatomy reveals that cerebral structure abnormalities connected to the motor and pre-motor areas of cerebral cortex, thalamus, red nucleus of midbrain and spinal cord. It has been reported that BRD2 is plays important role in the development of central nervous system **(14).**

BRD2 mRNA was detected in human brain including cerebral cortex, cerebellum, medulla, occipital cortex, frontal cortex, putamen, brain vesicles, neural tube, spinal cord, dorsal root ganglion in the anatomical context of the gene concern. BRD2 is a putative developmental transcription regulator expressed in brain and may be involved in the JME cortical microdys-genesis **(5)**. Neuropathology of some patients with JME reveals increased number of and diffusely distributed dystopic neurons in gray matter stratum molecular and subcortical white matter of brains **(15)**.

In 2003, Pal et al. showed that SNPs within the *BRD2* (RING3) gene which is located at 6p21.3 might be susceptibility alleles (odds ratio 6.5) for autosomal recessive JME in families from New York (5). Linkage disequilibrium found two strongly JME-associated SNP variants in the promoter region of *BRD2* and a common variant haplotype in over 50% of 20 probands from families that had produced positive LOD scores for 6p21 during linkage analyses **(5)**. Fourteen patients (18.6%) had a positive family history of epilepsy. The 61 JME (81.3%) patients met the criteria for classic JME.

JME in *Cx-36* (connexin36) **[16]**. BRD2 deficient embryonic fibroblast cells more observed to proliferate more slowly than controls (Shang et. Al., 2009) and observed enhanced levels of cell death in brd2 deficient embryos. BRD2 is essential for chromatin structures and transcription during mammalian embryogenesis and neurogenisis. Over expression of BRD2 gene leads to neuronal degeneration and as gene predicts positively regulate the apoptosis of neurons. Neuronal programmed cell death during embriogenesis and development of CNS is 70% to 80% at various stages for the morphogenetic events. Multi generational JME families that had originally been genetically linked to chromosome 6p21.3 (Human leukocyte antigen [HLA] region) and associated with HLA alleles, as in New York families of European origin **(16,17).** In the recent study by Pal and colleagues analyzed 20 single nucleotide polymorphism (SNPs) in 20 probands in the chromosomal loci on 6p21 region associated with JME and found strong genetic relations with eight SNPs in BRD2 gene. The effect of BRD2 SNPs on promoter function is currently unknown, but they may alter the timing, tissue structure or level of Brd2 expression **(7)**. BRD2 gene between alternative exon 2 and exon 3 were shows highly polymorphic microsatellite and certain alleles were strongly associated with JME (Green berg et al., 2000),

CONCLUSION

In conclusion, we identified six nonsense mutations in BRD2 in 4 males and 2 females and molecular genetic analysis of BRD2 is important for a definite diagnosis of JME. The relationship between the genotype and clinical phenotype is not known. Further large scale of Familial and twin studies are required to investigate for strong involvement of BRD2 and BRD3 genes in the genetic susceptibility of JME syndrome. However, the exact impact of the mutation needs to be confirmed by future studies on function.

Conflict of interest statement

No conflict of interest to disclose.

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