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Novel missense mutations in BRD2 gene of JME patients: A study from South India

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Abstract

Background: The genetic background for Juvenile myoclonic epilepsies (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. JME inheritance is autosomal dominant in all BRD2 mutations show heterozygosity in affected individuals. The JME is estimated around 3 in 10,000 with peak age at 14.5 to 15.5 years that affect both genders. Juvenile myoclonic epilepsies are primarily genetic in origin and genetically determined adolescent syndrome among the idiopathic generalized epilepsies (IGE) and almost certainly involve multiple genes. In the current study direct sequencing of the BRD2 gene exhibited a heterozygous missense mutations (C>A, A>T, G>A) in exon 11-12 that may alter the amino acid there by the function of the protein molecule.

Objectives: 1. To support innovative research, of the highest scientific merit, that has the potential for patient benefit; 2. To identify the mutations in BRD2 gene of JME patients

Methods: The case-control study design was used to test the potential involvement of BRD2 gene variations in the etiology of JME. We performed molecular a molecular screening of BRD2 gene exonic sequences for the detection of mutations by genomic PCR amplification and direct sequencing through ABI PRISM® 377 DNA Analyzer.

Result: Missesnse mutations were observed in exons 11and12 of BRD2 gene in unrelated JME cases from south Indian population.

Conclusion: We found 3 novel missense mutations of Bdr2 gene in 2.2% of unrelated JME patients from south India. The present study is the first report in relation to JME and Brd2 gene from this part of the world. Large scale family studies are required to establish the present observation in different ethnic populations.

Keywords: BRD2 gene, JME, M.J, messense mutation, SNP, whole exone sequence

Introduction

Juvenile Myoclonic Epilepsy (JME) is one of the most common epilepsy syndromes accounting for 7% of all cases of epilepsy [1, 2], and is classified as one of the idiopathic epilepsies**.** Epilepsy affects 1% of people worldwide, in an estimated 40% of whom it is genetically determined [3]. The genetic back ground for JME account for 5-10% of all forms 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 (Radhakrishnan *et al;* 2000). About 7% of the siblings of probands with JME were observed to have epilepsy of which 30% will have JME [4].

Interictal and ictal EEG of JME patients is characterized by generalized spike-wave, multiple spikes and slow-wave discharges with normal background activity [5]. The major clinical characteristics of JME include myoclonic jerks and generalized tonic-clonic seizures, few patients may also suffer from absence of seizures [6, 5]. The activation of certain neural regions believed to disrupt the massive neural discharge that leads to epileptic seizures. And this electrical activity in the neuron and neuronal electrical discharges are believed to be regulated by ion channels.

Brd2 gene encodes a transcriptional regulator that belongs to the bromodomains and extra terminal domain (BET) family of proteins shown to be localized in human brain. The BRD2 gene maps to the major histocompatability complex (MHC) class II region on chromosome 6p21.3. [7]. Several BRD2 mutations were shown to be heterozygous in JME affected individuals exhibiting autosomal dominant inheritance [8]. Few gene varients of BRD2 gene related with disorganized neuronal connectivity and neurocortical-hyperexcitability were noted in JME with autosomal recessive inheritance.

The identification of susceptibility genes is a great challenge in JME patients and the role for BRD2–a transcriptional regulates brain development. We attempted to identity the genetic association of JME with Bromodomain containing 2 proteins (BRD2) gene variants through molecular analysis in south Indian population.

Material and Method

Study conducted

The present study was carried out 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).

Recruitment of JME patients

The 75 unrelated JME patients in whom 44 males and 31 females were recruited from Ethnic Dravidian population of South Indian from three states of Andhra Pradesh (AP) and

Telengana (T.S) and Karnataka (Hyderabad-karnataka region). The d 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). All JME patients were selected based on the diagnostic evaluation made according to the classification of the International League against Epilepsy (ILAE) [9].

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 primary diagnosis of JME patients was based on EEG, CT/MRI and clinical findings under the supervision of Neurologist who was specialized in epilepsy. The sample included male and female.

Normal Healthy Controls

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 Anadhar Pradesh, Telangana and Karnataka (H-K region) states of South India. The controls subjects were selected with same gender and age from High school students, Junior college students, friends, and recruited agencies of unaffected persons. The controls had no history of neurological disease or family history of epilepsy. The participation rate was 100% in the present study.

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 and in case of minor participants consent from parents or guardians.

DNA extraction

A volume of 5 ml of venous blood were collected from all JME and control groups and transferred into EDTA (Ehylene diamine tetra acetic acid) vacutainers. 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 degree centigrade. 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 gene screening.

Methods

PCR Amplification

The BRD2 is amplified by using ten primers for 12 exons of the BRD2 gene. The primers used in this study were 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'CTTAGCGGGTTATG CTGGAC3'	5'CCGCTCAGTACTC CCAACAC3'	209 bp	59.4 and 61.4
BRD2/E2	5'TAAGCTTAACCACC TCACTAGG3'	5'CATCTACACTAGG CAGACCAC3'	496 bp	58.4 and 59.8
BRD2/E3	5'GGATCGGTAGTCTC CCTATAA3'	5'CTACCTGGATAAC ACCTTCAGT3'	321 bp	57.9 and 58.4
BRD2/E4-5	5'TCTTTATTGCTGTC TGTGTTCTCA3'	5'CCCAGAGGAAAT CCACAGAT3'	487 bp	57.6 and 57.3
BRD2/E6	5'AAGTGGGCTTGGA GTGACAG3'	5'CACCTAGGCTCCC ATCACTG3'	475 bp	59.4 and 61.4
BRD2/E7	5'GCTCTTCTTGTGGT GTCT3'	5'CTGTACAGAACA GTGAGACC3'	312 bp	53.7 and 57.3
BRD2/E8	5'GCTGGGTATGTAGG GCACTG3'	5'CCCAATAAAAACT TTCAAGAGTGA3'	338 bp	61.4 and 55.9
BRD2/E9	5'CATGCCCTTTGTCC TCATTT3'	5'CATCCCCCAGAG AGACAGAA3'	399 bp	55.3 and 59.4
BRD2/E10	5'TTTTGCTGACAACT CTTTTTGC3'	5'AGACCCCACCAT CTTTCCTC3'	396 bp	54.7 and 59.4
BRD2/E11-12	5'GGGGCCCATAATAA GATGCT3'	5'GTCTAGGGGTCC GGTCCTG3'	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 for 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).

Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to examine the quality and quantity of extracted DNA the amplified PCR products were separated by electrophoresis using 2% agarose gel run at 110V for 15 minutes. 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.

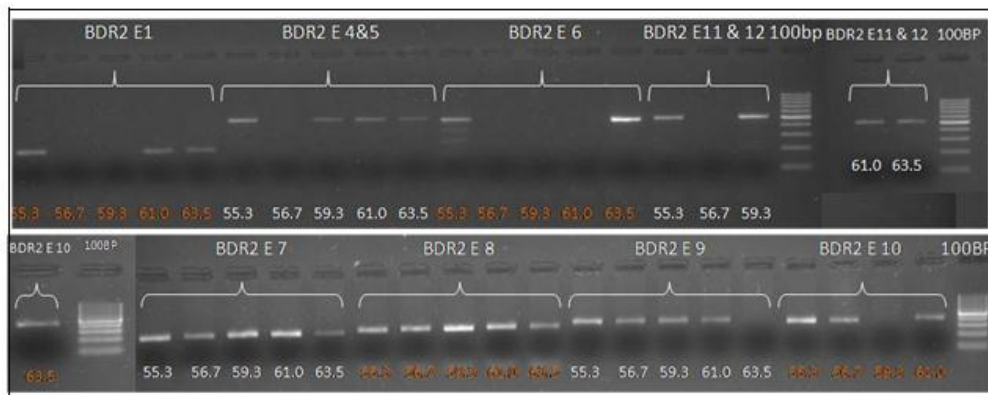


Fig 1: Gel picture showing the amplification produces of 12 exons of 3 JME patients

Result

The complete coding sequence of Brd2 gene is amplified from genomic DNA using standard protocols. We randomly selected three samples out of 75 JME patients and five healthy subjects for sequencing initially all the 12 exons of the gene. Based on these results, it was decided to process the remaining 72 JME samples and the 95 normal healthy control samples for sequencing the 11th -12th exons only to identify the mutations.

The three samples revealed an allelic variant in observed (2 females and 1 male).

The sequencing analysis of BRD2 gene was screened utilizing double strand conformational analysis (DSCA) by using ABI 377 automated sequences instrument and sequenced according to the ABI Big Dye Terminator Cycle sequencing protocol. Representative results are given in the following figure. The details of the missense mutations are given in Table: 2.

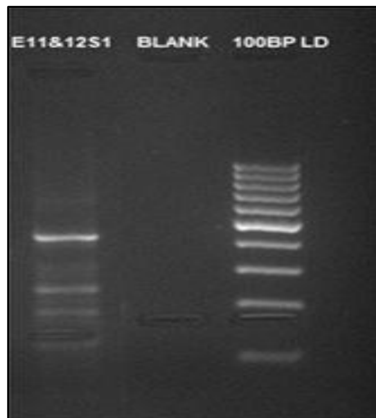
Fig 2: Representation of DNA sequencing eleectophreogram analysis of BRD2 gene

The following samples were used for primers standardization of Brd2 gene, using JME samples- SP-5075, SP-5285 and SP-5288 which was further used for whole-exone sequencing (WES) of BRD2 gene for from all the 1to12 exons in all 75 JME samples and 100 healthy controls.

Mutation analysis

Direct sequencing of all 12 exons of the BRD2 gene initially follows only in three JME patients revealed a heterozygous missense mutations in exon 11-12 and further sequencing on 72 JME patients in exon 11-12, which may result in three missense mutations (2.2%) seen in 2 males and 1 female in BRD2 gene.

Fig 3: Mutation sequencing analysis in exon 11-12 of BRD2 gene



A missense mutation changes that codon for one amino acid is replaced by a codon for another amino acid. This type of mutation is a change in one DNA base pair that results in the substitution of one amino acid for another in the protein made by a gene. The first phenotype allele was identified in whole-exone sequencing (WES) has revealed a base substitution in total number of 75 JME samples and lead to a different amino acid substation. The sequencing result revealed an allelic variant in the form of two missense mutations leads the amino acid substitution in exon 11 (C>A and A>T) and one missense allelic variant observed in exon 12 (G>A). An allelic variants found after sequencing analysis are presented in table: 2.

Table 2: Missense mutations association with BRD2 gene in JME patients

Base pair	Allelic variants	Position of Exon	Subtype of mutation	Aminoacid changes	Age at onset	Type of seizure	Gender
Bp141	CAC>AAC	Exon-11	Missense	His> Asx	15.5	Myoclonic	F
Bp283	AGA>TGA	Exon-11	Missense	Pro>Arg	16	Myoclonic	F
Bp536	GCG>ACG	Exon-12	Missense	Ala>Thr	26	GTCS	M

We found two missense mutations in exon 11 and one point missense mutation in exon 12 in three JME samples: SP-5288; SP-5136; and SP- 5052, and nucleotides at 141, 283, and 536 base pairs. 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).

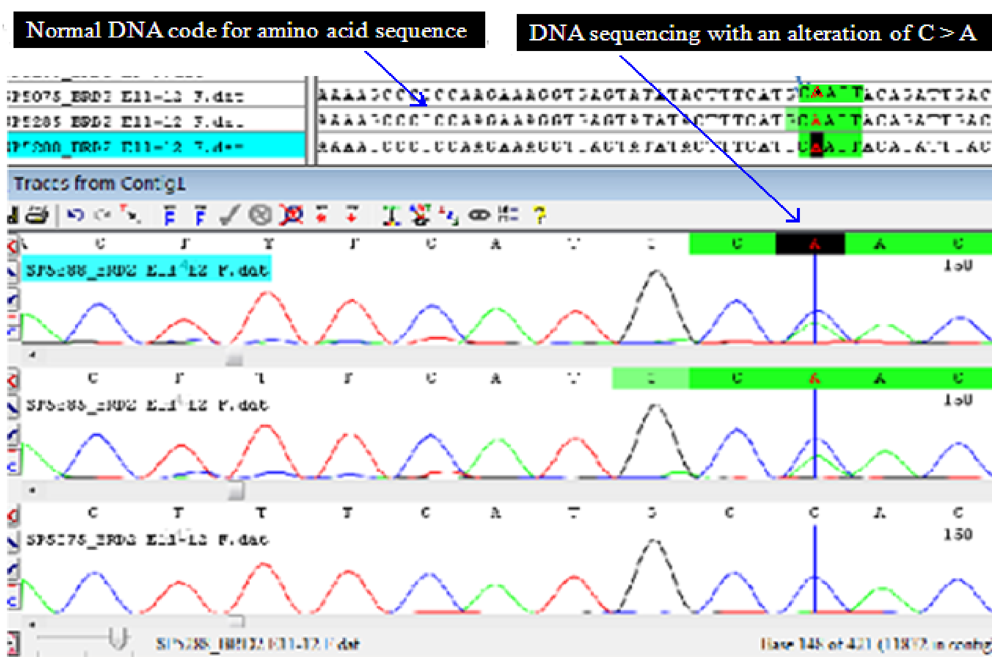


Fig 4: sequencing result showed a missense mutation in DNA sequencing with an alteration of C>A in exon 11.

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 [10]. Therefore, synchronized activity of large numbers of neurons abolishes their normal 'wakeful' functions. In a recent report of neuron-anatomy reveals that cerebral structure abnormalities are connected to the motor and promoter areas of cerebral cortex, thalamus, red nucleus of midbrain and spinal cord. It is known that Brd2 plays an important role in the development of central nervous system [11].

Table 3: Mutation association and gene-scan studies

Gene	Protein	Chromosomal locus	Polymorphism	Subjects	Phenotype
GABRA1	GABAa receptor alfa subunit	5q 32-35	(CA)n	63 families	32 JME
GABRA5	GABAa receptor alfa5 subunit	15q 11-13	(CA)n	94 families	72 JME
GRIK2	Ionitropic Gln R6	6q 16.3-21	(TAA)n	63 families	JME
CHRNA4	nAchR alfa4 subunit	20q 13	594 (C/T)	108 probands	60JME
SCNIA	Vol. gated Na+ chan. alfa1 subunit	2q 24	W1204R	165 families	72 JME
KCNQ2	Vol. gated K+ Channel Q2	20q 13.3	T752N	115 probands	71 JME
CACNI A1	Vol. gated Ca+ chan. alfa1A subunit	19q 13	(CAG)n	55 families	26 JME
EAAT2GLT(-)	Glutamate Transporter	11p 13-p12	603 (G/A)	130 families	57 JME
MAOA	Monoamine Oxidase typeA	Xp11.4-p11.3	(+30bp)	248 probands	126 JME
PAX6	Paired Box 6	11p13	(AC)m (AG)n	125 probands	70 JME
ARC	Activated regulated Cytoskeleton protein	8q 24.3	489 (C/T)	143 probands	81 JME
JPKIJH8 (+)	Jerky homologus	8q 24	N/A	10 families	5 JME
For GTCS	N/A	8p 11-12	N/A	88 families	52 JME
+	N/A	3q 26	N/A	130 families	95 JME
N/A	N/A	6 & 8	N/A	91 families	53 JME

N/A: Not available, bp: Base pair, Nucleotide given in brackets sources: www.nature.com/review/neuro May 2002, volume 3

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 microdysgenesis [4].

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 [12]. 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. Mutations in KCNQ2 and KCNQ3 genes, which control voltage gated potassium channels, have been identified in patients with benign familial neonatal seizures [13]. True pharmacoresistant forms account only for 10–15% of all JME cases who have uncontrolled seizures [14]. Patients with JME are described to have uncontrolled seizures if they continue to have >1 generalized tonic clonic seizure (GTCS) per year and/or >1 myoclonic jerk per month after starting of treatment with antiepileptic drugs [15, 16].

In the present study we observed novel nonsense mutations in the exons 11 and 12 in the patients in heterozygous condition but not in the normal healthy controls, suggesting the disease causing nature of these mutations. In 2003, Pal *et al.* showed that SNPs within the *BRD2* (RING3) gene present on chromosome 6p21.3 might serve as susceptibility alleles (odds ratio 6.5) for autosomal recessive JME families from New York [4].

Three susceptibility alleles have been associated with an increased risk of developing JME in *Cx-36* (connexin 36) [17].

The strong Linkage disequilibrium with two JME SNP variants in the promoter region of *BRD2* and a common variant haplotype in over 50% of 20 probands from families of European origin that had produced positive LOD scores for 6p21. 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), the seizure-related anatomical changes in the GABA system further supported BRD2's involvement in human IGE [18]. BRD2 deficient embryonic fibroblast cells were observed to proliferate more slowly than cells from controls, and enhanced levels of cell death in brd2 deficient embryos (Shang et. al., 2009). However, others reported that over expression of BRD2 gene leads to neuronal degeneration suggesting positive regulation of apoptosis of neurons by this gene. In general during embryogenesis at the time of CNS development 70 to 80 % of neurons are subjected to apoptosis at various stages for

marphogeni events. BRD2 is essential for chromatin structures and transcription during mammalian embryogenesis and neurogenisis. The effect of BRD2 SNPs on promoter function is currently unknown, but they may alter the timing, tissue structure or level of Brd2 expression [8]. The BRD2 gene expresses distinct tissue-specific transcripts that originate from different promoters and have strikingly different lengths of 5'UTR.

Conclusion
In conclusion, we identified three novel missense mutations through exon sequencing in exon 11 and 12 of BRD2 gene of *2.2%* of JME patients of south India, but in none of the normal healthy controls, suggesting the disease causing multigenetic involvement of mutations. Application of Bioinformatic tools may help in understanding the role of these missense SNPs in the function of protein. Large scale family studies in different populations are required to establish the role of these SNPs in JME further to develop diagnosis and therapeutic target.

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