Original Article

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Non-Synonymous Exonic Mutations and Polymorphism of BRD2 Gene in Juvenile Myoclonic Epilepsy

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Abstract

Background: Juvenile myoclonic epilepsies (JME) have a complex genetic disease and involved different susceptible idiopathic generalized epilepsy (IGE) genes in JME patients. 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. Non synonymous single nucleotide polymorphisms (nsSNPs) are coding variants that changes amino acid in their corresponding proteins, because nsSNPs can affect protein function. The discoveries of these genes, related to JME will provide the clinical practice and improve diagnosis and treatment of epilepsy.

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 will 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: Three missesnse mutations were observed in exon 7 of BRD2 gene in unrelated JME cases from south Indian population.

Conclusion: BRD2 gene polymorphism could significantly promising therapeutic targets in the prevention of JME. Loss of protein stability leads to loss of enzymatic activity of hyper neuronal excitability and possibly to the accumulation of the protein in cells.

Keywords: JME (Juvenile Myoclnic Epilepsy); BRD2 Gene (Bromodomain Containing 2 Protein Gene); missense mutation (A missense mutation may lead to the synthesis of a protein that is nonfunctional), nsSNP (non-synonymous Single Nucleotide Polymorphism)

Introduction

Juvenile myoclonic epilepsy (JME) is a common idiopathic generalized epileptic and genetically imparted syndrome that occurs in 5% to 10% of patients with epilepsy [1]. Mutations in one of several genes can cause or increase susceptibility to JME disease. The prevalence of JME in large cohorts has been estimated to be 2.8–11.9% of all epilepsies and 26.7% of genetic generalized epilepsies [2]. The number of genes implicated in epilepsy with the help of Next generation sequencing (NGS) technology. There is increasing evidence of cognitive dysfunction in these patients, with deficits reported on tests of frontal lobe function (Wandschneider

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etal., 2012). There are multiple susceptibility genes that participate in the genetic epistasis that produces JME, including malic enzyme 2, connexin 36, and bromodomain-containing 2 (BRD2), but many more are thought to be as yet unraveled [3]. JME often includes generalized tonic-clonic seizures and, less commonly, absence seizures. Some clinicians have refrained from calling this disease "benign" due to the need for often lifelong duration of treatment [4]. Impairment of cognition is a significant co-morbidity in individuals with JME and has a negative effect on activities of daily living [5]. Studies on the incidence of epilepsy in relatives of probands with JME, as well as on twins, have provided strong evidence for a genetic contribution [6,7]. SNPs in BRD2 (RING3) gene located on chromosome 6p21.3 have been found to be associated with the susceptibility of autosomal recessive JME cases among families from New York. The role for BRD2-a transcriptional regulator (RING3 belongs to a highly conserved subfamily of double bromodomain-containing proteins) is unclear in humans however, gene may result in disorganized neuronal connectivity and neocortical hyperexcitability [8]. The German authors recognized general precipitating factors, present in 28/47 cases, such as lack of sleep, sudden awakening and/or excessive alcohol intake [9]. BRD2 gene regulates the brain development and errors in regulation might explain the basis of development of JME. Prediction of the molecular effects of disease causing missense mutations by bioinformatics methods has been implemented in numerous recent studies. Association analyses have mostly been used to assess whether the frequency of specific alleles differs between JME patients and controls more than would be predicted by chance [10].

Material and Methods

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 study was carried out (2001-2014) in the Anatomy department, 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).

Interview and sample collection

The JME patients were interviewed in person with a standard questionnaire under the direction of epilepsy specialist. 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 had no history of neurological disease or family history of epilepsy. The participation rate was 100% in the present study.

Ethical approval

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 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. The extracted DNA 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

Primer/exons	Primer sequence Forward	Reverse	Amplicon Size (bp)	Annealing tempreture (C*)
BRD2/E1	5′CTTAGCGGGTTATG CTGGAC3′	5′CCGCTCAGTACTC CCAACAC3′	209bp	59.4 and 61.4
BRD2/E2	5′TAAGCTTAACCACC TCACTAGG3′	5′CATCTACACTAGG CAGACCAC3′	496bp	58.4 and 59.8
BRD2/E3	5′GGATCGGTAGTCTC CCTATAA3′	5′CTACCTGGATAAC ACCTTCAGT3′	321bp	57.9 and 58.4
BRD2/E4-5	5′TCTTTATTGCTGTC TGTGTTCTCA3′	5′CCCAGAGGAAAT CCACAGAT3′	487bp	57.6 and 57.3
BRD2/E6	5′AAGTGGGCTTGGA GTGACAG3′	5′CACCTAGGCTCCC ATCACTG3′	475bp	59.4 and 61.4
BRD2/E7	5′GCTCTTCTTGTGGT GTCT3′	5′CTGTACAGAACA GTGAGACC3′	312bp	53.7 and 57.3
BRD2/E8	5′GCTGGGTATGTAGGGCACTG3′	5′CCCAATAAAAACT TTCAAGAGTGA3′	338bp	61.4 and 55.9
BRD2/E9	5′CATGCCCTTTGTCC TCATTT3′	5′CATCCCCCAGAG AGACAGAA3′	399bp	55.3 and 59.4
BRD2/E10	5′TTTTGCTGACAACT CTTTTTGC3′	5′AGACCCCACCAT CTTTCCTC3′	396bp	54.7 and 59.4
BRD2/E11-12	5′GGGGCCCATAATAA GATGCT3′	5′GTCTAGGGGTCC GGTCCTG3′	489bp	57.3 and 63.1

Table 2. Cycle sequencing PCR mixture constituents for BRD 1 Marker

S. No.	Components	Volume(µl)
1	PCR Buffer(5X)	1
2	MgCl2 (25mM)	0.8
3	DNTPs(200mM)	0.6
4	Primer F(10pM/µl)	0.23pL
5	Primer R(10pM/ µl)	0.23pL
6	Taq Polymerase	0.5
7	Molecular Biology grade water	2.64
8	DNA (10ng/ µl)	4
		Total: 10.0 pL

Only one of the primers i.e. either forward or reverse primer was used during cycle sequencing

Table 3. Cycle Programme sequencing conditions for BRD 1 Marker

Step	Process	Temperature	Time
1 Cycle	Initial denaturation	95° C	1-3 min
35 cycle	Denaturation	95° C	3 min
	Annealing	(primer dependent)	20-45 sec
	Elongation	72° C	1 min
1 Cycle	Final extension	72° C	4 min
Final step	Hold	5° C	Indefinite

The annealing temperature is primer is dependant and varies for each primer

extracted DNA samples were stored in freezers at -72°C until further use for gene screening.

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 genomic, Banglore.

DNA sequencing method

PCR in presence of deoxynucleotide phosphate (ddNTPs), (enzymatic method) developed by Sanger's chain termination method. Genetic information is stored in the order or sequence of nucleotides in DNA chain termination sequencing and is the standard method for the determination of nucleotide sequence. DNA to be sequenced is prepared as a single-strand molecule, which acts as a template for DNA synthesis.

Agarose Gel Electrophoresis

It is used to examine the quality and quantity of extractedDNA 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.

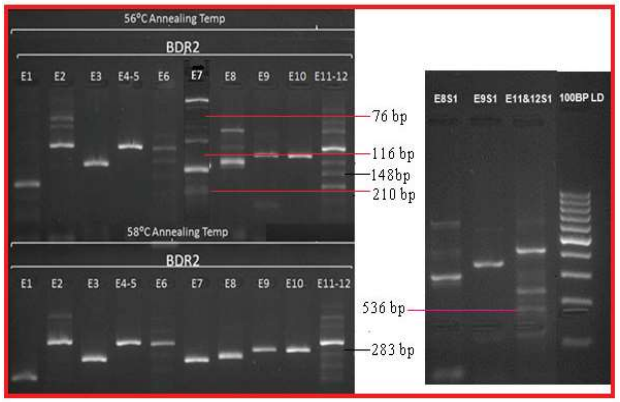

Fig. 1: Gel image showing banding pattern of BRD2 in exon 7 revealed c.3150 G>A at 74 bp, c.8919 G>A at 166 bp, and c.3753 G>C at 210 bp polymorphisms.

Table 4: Missense mutations associated with 7 exon of BRD2 gene in JME pateints

Subtype of Mutation	Nucleotide variatns	Protein designation	Aminoacid changes	Age at onset	Type of seizures and sex
Missense	c.3150 CGG>CAG	p.Arg74Gln	Arginine to Glutamine	18	GTCS & Male
Missense	c.8919 AGA>AAA	p.Arg166Lys	Arginine to Glutamine	16	MJ & Male
Missense	c.3753 AGA>ACA	p.Arg210Thr	Arginine to Thyrosine	15	MJ & Male

Result

Identification of missense mutation

We performed a molecular investigation by direct sequence of all twelve exons of BRD2 gene, by using four JME samples and ten healthy controls used for complete coding sequence of BRD2 gene. This molecular analysis revealed novel mutations in heterozygous form in the 7th exon that caused for an amino acid substitution.

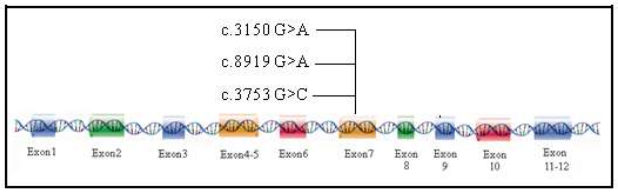

Fig. 2: Scheme to showed sequencing result a missense and nonsense mutation in DNA sequencing with an alteration of Arg>Gln, Arg>Lys and Arg>Thr observed, exonic polymorphism in exon7

Polymorphisms

Three SNPs were detected in BRD2 exon 7 and two mutations are transitions c.8919G>A, c.3150G>A, and one mutation is transversin c.3753G>C at 116, 76 and 210 base pair substation. An allelic variant found after sequencing analysis and representative results are given (Table 4). The allelic variants occur in the conserved exons, the consequence may be serious, results the pathogenic mutation.

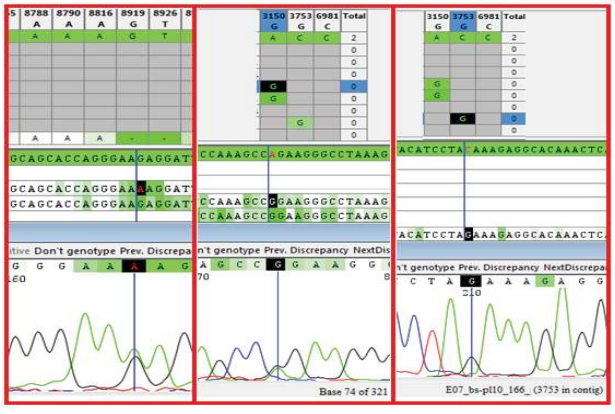

Fig. 3: Sequencing results of BRD2 gene in exon 7 revealed three missense mutation, changes the nucleotide substitution c.3150 CGG >CAG at 166 bp, c.8919 AGA>AAA at 74 bp and c.3753 AGA>ACA at 210 bp heterozygous polymorphisms.

Missense mutation affecting amino acid sequence of the predicted protein

Genetic variation of single base changes in 7th exon of the BRD2 gene can affect its expression or the function of its protein products. There is no such mutations were found in the 100 normal healthy subjects. These mutations have not been previously reported in the NCBI data base (SNPs). Non-synonymous exonic mutations (nsSNPs) lead to altered amino acid that may affect protein function.

A missense variant is a single base change in a coding region that causes an amino acid change in the corresponding protein; the change can cause drastic phenotypic consequences. Amino acid substitutions in exons may evolutionarily conserved residues and can be good candidates for true pathogenicity. However, a change in the amino acid sequence in JME patients caused by change of one base-pair gives rise to serve alternation in the biological function.

Discussion

Central nervous system associated with BRD2 (RING3) gene

In recent report investigate in animal model revealed that cerebral structure abnormalities connected to the motor and premotor areas of cerebral cortex, thalamus, red nucleus of midbrain and spinal cord. In animal model Brd2 gene is playing important role in development of central nervous system [11]. BRD2 has a putative nuclear transcriptional regulator gene, which plays an important function in the development of the central nervous system in humans.

The cerebral structural abnormalities during brain development in JME patients may result in disorganized neuronal connectivity and regions of neocortical hyperexcitability, leading to clinical seizures.

The human BRD2 gene has been strongly linked and associated with JME risk and electroencephalographic abnormalities. The

human BRD2 gene has been shown to play a critical susceptibility role in a common form of JME, neural phenomena including abnormal EEG patterns [12,13]. The structural organization of the introns and exons of the human and mouse genes has been highly conserved. Epilepsy mutations affect proteins that regulate action potentials and synaptic function, both of which underlie neuronal communication. 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 reports disclosed the over expression of gene leads to neuronal degeneration suggesting positive regulation of apoptosis of neurons by BRD2 gene. In general during embryogenesis at the time of CNS development 70 to 80% of neurons are subjected to apoptosis at various stages for marphogenic events. BRD2 is essential for chromatin structures and transcription during mammalian embryogenesis and neurogenisis. A few SNP alleles in BRD2, Cx-36, and ME2 were reported waiting still confirmation by other large cohorts [14]. Recently the contribution of copy number variants was also disclosed in a minority of JME population. It was striking to note that the existing evidence supported a neuro-developmental origin for common epilepsies like JME [15].

The effect of BRD2 in single nucleotide polymorphism on promoter function is currently unknown, but they may alter the timing, tissue structure or level of expression [16]. The BRD2 gene expresses distinct tissue-specific transcripts that originate from different promoters and have strikingly different lengths of 5'UTR. Neurodegeneration leads to the loss of anatomy and physiology of the nervous system. Dysfunction of Purkinje cells can lead to lack of motor coordination (ataxia), a characteristic symptom of many debilitating movement disorders (Ito, 1984).

Neuronal programmed cell death during embriogenesis and development of CNS has estimated 70% to 80% at various stages for the morphogenetic events. 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 [17]. A recent study investigating patients with JME and their siblings may indicate more global, genetically determined pattern of cognitive deficits [18].

Conclusion

BRD2 is the part of the Bromodomain and Extra-Terminal (BET) protein. We found three missense mutations in BRD2 gene in unrelated JME patients. Based on our results we suggest that BRD2 gene is more prone to mutations in JME than the other IGE. The hypothesis supported by our findings shows three pathological mutations in exon-7. The large scales of family studies in different populations are required to establish the genetic role of these SNPs in JME patients and further to develop diagnosis and therapeutic target.

Conflicts of interest

The authors have no conflict of interest to declare.

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