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Confirmation of R82Q Mutation in g2 Subunit of Gamma Amino Butyric Acid Receptor in an Iranian Family

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Abstract: In this study, 46 patients with Febrile Seizures were selected and total DNA was extracted from white blood cells. All exons of GABRG2 gene were amplified by standard PCR. After which the PCR products were screened by Single Stranded Conformational Polymorphism (SSCP) analysis. Selected samples in SSCP analysis that showed new conformer were sequenced. In order to verify the sequencing data, a restriction analysis was carried out. We report R82Q mutation in GABRG2 gene in an Iranian family. Proband was a 12 years old boy. SSCP and Sequencing of exon-2 of GABRG2 gene in proband demonstrated an exonic G245A mutation leading to R82Q substitution. The RFLP of PCR product showed heterozygosity in patients. Arg82 is a conserved amino acid in the benzodiazepine binding site at the N-terminus of the g2 subunit. The R82Q mutation does not alter the response of the receptor to Gamma Amino Butyric Acid but impairs the potentiation by benzodiazepine. Result of this research and several other lines of evidence support complex inheritance, heterozygosity and diverse phenotypes for epilepsy.

Key words: Febrile seizure, GABA receptor, R82Q mutation, molecular screening

INTRODUCTION

Febrile Seizures (FS) are the most common convulsive syndrome in childhood. Genetic components are significantly involved in the etiology of the majority of FS (Hirose *et al*., 2000; Hirose *et al*., 2002a, b; Kaneko *et al*., 2002). The incidence of FS reaches 6-9% in the Japanese population while it is 5% in Caucasians (Kugler and Johnson, 1998). This racial difference clearly suggests that genetic components are involved in the etiology of FS. Several genetic loci for FS have been mapped and genetic defects have been identified in Autosomal Dominant Epilepsy and Febrile Seizures (ADEFS) (Ito *et al*., 2002) and Generalized Epilepsy with Febrile Seizures (GEFS) (Scheffer and Berkovic, 1997). Gamma Amino Butyric Acid receptors are ligand-gated ion channels that have always been an inviting target in the etiology of epilepsy. Gamma Amino Butyric Acid (GABA) is the major inhibitory neurotransmitter in the CNS. Mutations in the gene encoding the g2 subunit of Gamma Amino Butyric Acid-A receptors, GABRG2 have been identified in four families with generalized epilepsy syndromes associated with febrile seizures, including GEFS and classical Childhood Absence Epilepsy (CAE) (Baulac *et al*., 2001; Wallace *et al*., 2001; Harkin *et al*., 2002; Kananura *et al*., 2002). Moreover, mutations in GABRG2 have been found as a cause of both ADEFS and FS followed by CAE. The missense mutation 983A.T: K328M was first identified in a French family and affected individuals showed the ADEFS phenotype (Baulac *et al*., 2001). A second missense mutation (c.245G.A: R82Q) was found in an Australian family in which affected individuals showed FS and CAE (Wallace *et al*., 2001). In this study we searched for these previously identified mutations in Iranian patients with FS and related epileptic disorders. Approximately seven hundred thousand individuals are affected by epilepsy in Iran, making this disorder a very economically and socially important issue to address. In addition, there is no definite statistical data about portion of FS and related epileptic disorders. The main objective of this study is the optimization of a method for screening of genetic alterations involved in pathogenesis of FS. Molecular screening of previously identified mutations in the patients with epileptic disorders is a sound approach for differential diagnosis in future.

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MATERIALS AND METHODS

Patients and samples: In this study, a total of 46 patients with FS, registered in Iran Epilepsy Association, were selected. The purpose of the study was explained to the subjects and information recorded with their consent. The Institutional Ethics Committee had approved the study protocol. Genetic counseling was carried out with patients and their families. Family pedigree for patients was designed by personally interviewing the subjects and their family members (parents or siblings). For each patient; clinical and informative data was collected and recorded. Epilepsy was diagnosed and classified according to the definitions and terminologies of ILAE classification of seizures and epileptic syndromes (Commission on Classification and Terminology of the International League Against Epilepsy, 1989). All patients were examined neurologically for detection of abnormalities such as mental and cranial nerves as well as long tracts and cerebellar functions. Patients who had post-ictal motor paralysis were not included in this study. Laboratory tests showed no signs of any serious viral or microbial infections such as meningitis. Electroencephalography was carried out for each patient to detect special causes of epilepsy. Patients with abnormal neurological findings compatible with brain disorders and mentally-retarded children were excluded. Epileptic patients whose conditions were affected by meningitis were also excluded. Magnetic Resonance Imaging (MRI) was requested for some patients in situations where doubtful brain malformations were suspected and those detected with any were subsequently excluded. Finally, peripheral blood sample was collected from patients and their families.

DNA extraction and PCR amplification: Total DNA was extracted from white blood cells. Forward and reverse primers were designed for all exons of GABRG2 gene and DNA amplification was carried out in standard conditions. For the design of primers and definition of mutations, we used the nomenclature of the cDNA (refer to the full length GABRG2 cDNA in GenBank with accession number NM_198904). Sequences of primers are available upon request.

SSCP and sequencing: The amplified DNA fragments were initially screened by analysis of Single Stranded Conformational Polymorphism (SSCP). Ten microliter PCR products were added to 10 μL SSCP buffer containing 2 μL TBE 1X and 7 μL formamide loading buffer (Sambrook and Russell, 2001) and 2 μL NaOH 0.05 M. The reactions were denatured at 96EC for 10 min, ice-chilled and separated by electrophoresis in 10% acrylamide-bisacrylamide (29:1) gels containing 5% glycerol without urea. TBE 1X was used as running buffer. Voltage conditions were optimized as described by previous studies (Sunnucks *et al*., 2000). The samples selected in SSCP analysis were sequenced by ABI 3700 sequencer (Gene Fanavaran, Macrogen, South Korea).

Restriction fragment length polymorphism: In order to confirm of sequencing data and detection of R82Q mutation Restriction Fragment Length Polymorphism (RFLP) was carried out. Five microliter of the PCR product was incubated with 4 units of *Hae*III in presence of 2 μL enzyme specific buffer (10x) and 1 μL BSA for 16 h at 37EC. Digestion mixture was studied in a poly acrylamide gel electrophoresis.

RESULTS

R82Q mutation was detected in a 12 years old male proband with Febrile and Afebrile seizures from a family with febrile seizure. He had experienced three generalized tonic-clonic seizures and was on AED. The historical study of the proband and his parents showed some symptoms in his mother. She had suffered from eyelid myoclonia and minor epileptic seizures. PCR-SSCP showed a new conformer for DNA samples of patient and his mother (Fig. 1). Sequencing of the forward and reverse strands of these fragment demonstrated an exonic G245A mutation leading to R82Q substitution (Fig. 2). The nucleotide substitution (G6A) at this position changes a

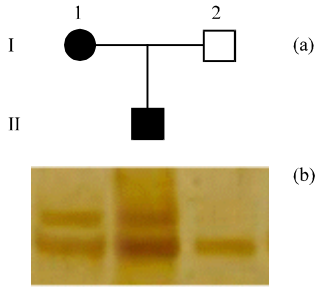

Fig. 1: (a) Pedigree of family; solid symbols in the pedigree refer to patients with epilepsy (I1 for mother, I2 for father and II1 for son). (b) SSCP pattern of the proband and his parents for exon-2 of GABRG2. SSCP analysis showed new conformers in patients (I1 and II1). Denatured products were run in 10% acrylamide gel containing 5% glycerol and revealed by silver staining

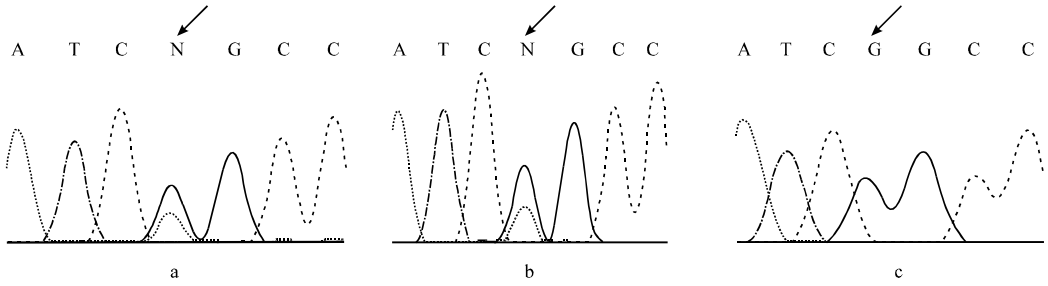

Fig. 2: Chromatogram of the G245A mutation: Traces of the sense DNA strands of the proband (a), his mother (b) and his father (c). The mutated nucleotide sequences are indicated over the sense strand by arrow

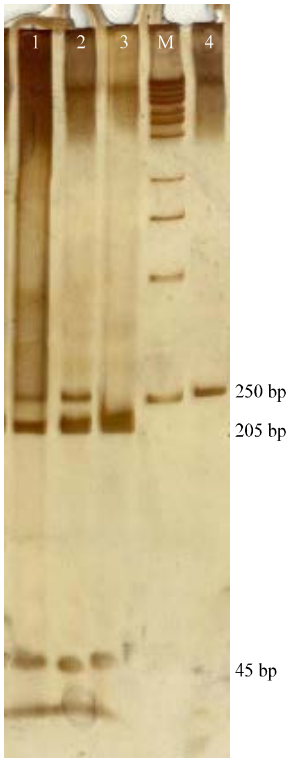

Fig. 3: Restriction Fragment Length Polymorphism (RFLP). PCR products were analyzed using *Hae* III endonuclease for digestion as described in Methods. The fragments were separated in an 8% polyacrylamide gel and bands were detected by silver staining method. The non-digested PCR product (lane 4) is 250 bp in length and the fragments separated from the GGCC restriction site are 205 and 45 bp long. G245A changes this restriction site and interferes with the action of enzyme. Therefore, heterozygous individuals harbouring the G245A mutation (lane 1: mother, lane 2: son) have three fragments of 250, 205 and 45 bp. The father of family (lane 3) with homozygous wild type alleles showed normal pattern of RFLP. (M: DNA size marker)

restriction site. This site is recognized in wild type sequence by the *Hae*III endonuclease that splits the 250 bp PCR product into 205 and 45 bp fragments. The RFLP of PCR product showed heterozygosity in mother and her son and generated 250, 205 and 45 bp fragments (Fig. 3). Analysis of this region in father of family did not show any mutation and the enzymatic digestion produced 205 and 45 bp fragments. These results showed a typically autosomal dominant inheritance for G245A mutation in this family.

DISCUSSION

We report a family with FS with indications of a generally autosomal dominant inheritance. On the other hand, clinical symptoms were different between the proband and his mother, as the latter did not experience febrile convulsions during childhood. Thus, it remains possible that either two different epilepsy phenotypes cluster in this small family or it is a clinical heterogeneity (two phenotypes with one genotype). R82Q mutation was detected in this family; however, the origin of this mutation in mother is unknown. Unfortunately, the grandparents of proband were inaccessible. the sequencing and RFLP analysis showed that R82Q mutation in this family follows an autosomal dominant inheritance and this mutation has been inherited to the boy from his mother.

The GABAA receptor is a ligand-gated Cl^- channel and serves as the main inhibitory system in the brain. The receptor functions as a heteropentamer consisting of several subunits including a, b, g, 1, u, p and r. Furthermore, each subunit has several subtypes. Since a1, b2 and g2 are expressed widely in the brain, (a1)2(b2)2(g2) is hypothesized to be the main configuration of GABAA receptors in the brain (Hirose *et al*., 2002b). The GABAA receptor is the target of many neuronal inhibitory drugs such as benzodiazepines and barbiturates. The g subunit of GABAA receptors has binding sites for benzodiazepine at the N-terminus and spans the plasma membrane four times (Wong and Snead, 2001). Arg82 is a conserved

amino acid in the benzodiazepine binding site at the N-terminus of the g2 subunit. The electrophysiological properties of GABAA receptors bearing R82Q mutations have been characterized; this mutation does not alter the response of the receptor to GABA but impairs the potentiation by benzodiazepine (Baulac *et al*., 2001). GABAA receptors with the R82Q mutant g2 subunit are considered to be insensitive to endozepine, a putative endogenous benzodiazepine-like substance. Mutant GABAA receptors bearing the R82Q mutation may not be able to stabilize neuronal excitabilities, thereby loosing control by endozepine. In addition, functional studies in oocytes *in vitro* showed that this mutation is associated with either reduction or abolition of the GABA-activated Cl^- current or loss of sensitivity to benzodiazepines (Baulac *et al*., 2001; Wallace *et al*., 2001; Harkin *et al*., 2002). We have reported a family with two types of epilepsy in which members are affected by the same mutation. Despite previously identifying of R82Q mutation in large families with FS, such families still provide support for complex inheritance in more complicated phenotypes. For example, in one GEFS family in which the proband has Severe Myoclonic Epilepsy type I (SME-I), there is clinical genetic evidence to indicate that at least three epilepsy genes could be inherited (Baulac *et al*., 2001; Harkin *et al*., 2002). Incomplete penetrance in addition to variable expressivity and polygenic inheritance are main features of epilepsy (Szepetowski and Monaco, 1998). Interestingly, affected individuals in one of the two families with $GEFS^+$ presented typical CAE symptoms together with the occurrence of febrile convulsions (Wallace *et al*., 2001). In the light of these findings and the uncertainties, which are sometimes associated with the diagnosis of FS, it is possible that the R82Q mutation reported here has a pivotal role in epilepsy in this family.

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