A Splice-Site Mutation in GABRG2 Associated With Childhood Absence Epilepsy and Febrile Convulsions

Colette Kananura, MS; Karsten Haug, MD; Thomas Sander, MD; Uwe Runge, MD; Wenli Gu, MS; Kerstin Hallmann, MS; Johannes Rebstock, MD; Armin Heils, MD; Ortrud K. Steinlein, MD

**Context:** Missense mutations in the GABRG2 gene, which encodes the γ2 subunit of central nervous γ-aminobutyric acid (GABA)A receptors, have recently been described in 2 families with idiopathic epilepsy. In one of these families, the affected individuals predominantly exhibited childhood absence epilepsy and febrile convulsions.

**Objective:** To assess the role of GABRG2 in the genetic predisposition to idiopathic absence epilepsies.

**Design:** The GABRG2 gene was screened by single-strand conformation analysis for mutations. Furthermore, a population-based association study assessing a common exon 5 polymorphism (C588T) was carried out.

**Patients:** The sample was composed of 135 patients with idiopathic absence epilepsy and 154 unrelated and ethnically matched controls.

**Results:** A point mutation (IVS6+2T→G) leading to a splice–donor site mutation in intron 6 was found. The mutation, which is predicted to lead to a nonfunctional protein, cosegregates with the disease status in a family with childhood absence epilepsy and febrile convulsions. The association study did not find any significant differences in the allele and genotype frequencies of the common exon 5 polymorphism (C588T) between patients with idiopathic absence epilepsy and controls (P>.35).

**Conclusions:** Our study identified a splice–donor-site mutation that was probably causing a nonfunctional GABRG2 subunit. This mutation occurred in heterozygosity in the affected members of a single nuclear family, exhibiting a phenotypic spectrum of childhood absence epilepsy and febrile convulsions. The GABRG2 gene seems to confer a rare rather than a frequent major susceptibility effect to common idiopathic absence epilepsy syndromes.

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**CHILDOOD ABSENCE epilepsy (CAE) is one of the most common subtypes of idiopathic generalized epilepsy (IGE). It is characterized by daily clusters of absence seizures at an age of onset between 2 and 12 years.**

Febrile convulsions (FCs) are the most common seizure subtypes, affecting about 3% to 5% of children younger than 6 years. While CAE is often followed by other IGE syndromes, including generalized tonic-clonic seizures and myoclonic seizures, only 3% to 7% of children suffering from FCs develop epilepsy later in life. A genetic basis for both CAE and FC is well established. Because the incidence of FC is significantly increased in patients with CAE (10%-15%) compared with the general population, a genetic overlap between both of these disorders has been suggested. Such a common molecular basis is most obvious in the syndrome of “generalized epilepsy with febrile seizures plus” (GEFS+), a monogenic or oligogenic epilepsy that was first described in 1997. Generalized epilepsy with febrile seizures plus is characterized by FCs that may persist beyond the age of 6 years and are often followed by generalized seizures, including myoclonic and absence seizures.

While the molecular basis of common forms of seizure disorders, including FC and CAE has been elusive so far, disease-causing GEFS+ mutations have already been identified in SCN1B, SCN1A, SCN2A, and GABRG2, genes encoding neuronal sodium channel subtypes and the γ2-subunit of central nervous γ-aminobutyric acid (GABA)A receptors, respectively. A potential
PATIENTS AND METHODS

PATIENTS

The study sample included 135 unrelated German patients with IAE at the University Hospital Rudolf Virchow at the Free University of Berlin (Berlin, Germany) and at the University Hospital of Bonn (Bonn, Germany). The sample consisted of 59 patients with juvenile AE and 46 patients with CAE who had at least 1 first-degree family member affected by IGE. In addition, 19 patients with juvenile AE and 11 with CAE were included as sporadic cases. The study protocol was approved by the local ethics committees, and written informed consent was obtained from all participants. Diagnostic criteria for IAE (CAE or juvenile AE) were: (1) onset with typical absence seizures; (2) age at onset of typical absence seizures between 3 and 20 years; (3) electroencephalogram (EEG) findings of normal background activity and paroxysmal spike-wave EEG discharges; and (4) normal intellectual and neurological status apart from seizures. Exclusion criteria included (1) evidence for structural lesions or metabolic or degenerative diseases of the brain; (2) atomic/astatic or tonic seizures; (3) complex partial seizures; (4) epilepsy with myoclonic absences; and (5) exclusively stimuluss-induced seizures. In case of a rare mutation in the sample of patients with IAE, we assessed the presence of the latter in a sample comprising 88 unrelated and ethnically matched controls. For the association study, we obtained the genotype and allele frequencies in all 135 patients as well as in 154 unrelated and ethnically matched controls. All controls were healthy volunteers of German descent.

FAMILY 510

The 15-year-old index patient (II-1) had exhibited typical pyknoelectic absence seizures starting at the age of 4 years (syndrome diagnosis: CAE) and experienced 3 uncomplicated FCs at age 4 years. In addition, he had 1 generalized tonic-clonic seizure at age 10 and 1 at age 11 years. His interictal EEG results showed 3 s generalized spike-wave discharges during resting as well as photosensitivity. At age 12 years, he began daily treatment with 1800 mg of valproic acid and had been free of seizures since that time. His 13-year-old sister (II-2) had 4 uncomplicated FCs at age 3 years. She exhibited typical absence seizures and several generalized tonic-clonic seizures at age 4 years (syndrome diagnosis: CAE). Results of her interictal EEG showed 3 s generalized spike-wave discharges while resting. Valproic acid treatment was started and she had been seizure-free since then. The 42-year-old father (I-1) had experienced 20 uncomplicated FCs between the ages of 3 and 6 years. From ages 6 to 15 years, he was treated with phenobarbital and ethosuximide and remained seizure-free without antiepileptic treatment. He had no siblings and his parents had no known history of seizures. The 43-year-old mother (I-2) reported no history of epileptic seizures.

MUTATION SCREENING

Genomic DNA was extracted either from 10-mL aliquots of EDTA-anticoagulated blood samples or from lympho-

role of the GABAergic system has often been implicated in epileptogenesis, however, genetic evidence for this hypothesis has been obtained only recently by the discovery of different GABRG2 mutations identified in 2 families. The phenotype in one of these families was described to be compatible with GEFS+, but no further details regarding the seizure types observed in the affected pedigree members were given. In the second family affected, individuals predominantly had CAE and FC. Accordingly, these findings raise the question whether genetic variation of the GABRG2 gene confers susceptibility to the epileptogenesis of common subtypes of idiopathic absence epilepsies (IAEs). We therefore systematically searched for mutations in genomic DNA and performed a population-based association study assessing a frequent silent polymorphism (C588T) in exon 5 of the GABRG2 gene.

RESULTS

VERIFICATION OF THE GABRG2 WILD TYPE SEQUENCE

The GABRG2 cDNA sequence (XM_003986.2), which had been deposited into Genbank on April 6, 2001, differed from the previous sequence (XM_003986.1) by the presence of an additional guanine following nucleotide position 770, which is located at the boundary between exons 6 and 7. Compared with the previous GABRG2 sequence, the additional guanine would result in an in-frame stop codon following amino acid position 227 upstream of the first transmembrane domain. Reverse transcriptase PCR amplification of the respective region could not verify the extra guanine. These results were further confirmed by amplification from genomic DNA and direct sequencing of both the exon 6/intron 6 and intron 6/exon 7 boundaries (data not shown).

DETECTION OF A SPLICE–DONOR SITE MUTATION IN A FAMILY WITH CAE AND FC

The coding region and exon/intron boundaries of GABRG2 were screened by single-strand conformation analysis for mutations in genomic DNA samples derived from 135 patients. The DNA of the index patient (II-1) from family 510 showed aberrant electrophoresis patterns indicative of a sequence variation in the respective PCR fragment containing exon 6. Direct sequencing revealed a single base pair exchange at the splice–donor site of intron 6, substituting a thymine with a guanine (IVS6+2T→G) (Figure 1). No further mutations were found by screening the entire GABRG2 coding region.

For evaluating the segregation of the IVS6+2T→G mutation, exon 6 and the adjacent part of intron 6 were
blistoid cell lines, using a salting-out method. For single-strand conformation analysis, we designed specific primer sets amplifying all GABRG2 exons and adjacent exon-intron boundaries (primer sequences are available on request). Polymerase chain reactions (PCRs) were carried out in a PTC 200 (MJ Research, Waltham, Mass.), that contained a total 25-μL volume comprised of 30 ng of genomic DNA, 5 pmol each of forward and reverse primers, 200μM each of dinucleotide triphosphate, 1.5mM of magnesium chloride, 30mM of potassium chloride, 20mM of Tris hydrochloride (pH18.3), and 0.1 U of Taq DNA polymerase. Polymerase chain reaction parameters were as follows: denaturation at 95°C for 5 minutes followed by 33 cycles at 95°C for 30 seconds, annealing at 58°C to 64°C for 30 seconds, and extension at 72°C for 30 seconds followed by a final extension step of 5 minutes at 72°C. The obtained PCR products were denatured and run on 10% polyacrylamide gels for 14 to 16 hours at room temperature and at 4°C, respectively. After the run, the bands were visualized using a standard silver-staining protocol. Polymerase chain reaction products showing aberrant patterns were amplified again prior to direct sequencing with an ABI 377 sequencer (Applied Biosystems, Foster City, Calif). For verification of the mutation and screening of the control sample, we developed a restriction fragment length assay using primers n1005 (5’AGCTGACACTTCTCCATCTCACG) and Z n1070 (5’TAGAGGTATGTAGGAATCTCTCT) and n1066 (5’TGGACAGATCATCTGPAGGGTTA, n1025, 5’CAGGATAGGACGACATGAGTGT. Annealing temperatures were optimal at 65°C, and human brain RNA (Clontech, Palo Alto, Calif) was successfully used as template. Singleand nested-PCR attempts, however, failed to amplify the GABRG2 cDNA from total RNA derived from leukocytes. For the prediction of possible cryptic splice–donor sites within intron 6, the 1998 version of an online splice site predictor program was used (www.fruitfly.org).

EXON 5 POLYMORPHISM (C588T)

Exon 5 and the adjacent parts of introns 4 and 5 were amplified using primers n1065 (5’CCATCTTTATGT-TAATATCCTT) and n1066 (5’ACTGAGGTTAGG-GAGGATAC). Digestion of the PCR product with restriction endonuclease TaqI (MBI Fermentas) resulted in fragments of the following sizes: 99 bp + 36 bp + 17 bp + 6 bp (C-allele) and 91 bp + 36 bp + 17 bp + 8 bp + 6 bp (T-allele). The C588T polymorphism is probably identical to a previously described and incorrectly numbered exon 5 variant.13 Allele and genotype frequencies, χ² tests, power calculations, and the test for Hardy-Weinberg equilibrium were calculated using the SAS computer program (SAS Institute, Cary, NC). A 2-tailed type I error rate of 3% was chosen for the analyses.

Reverse Transcriptase (RT) PCR

Reverse transcription PCR was performed using the Titan One Tube RT-PCR-System (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s protocol. The following complementary DNA (cDNA) primers were designed for verification of the wild type GABRG2 sequence: n1024. 5’GGGCACACAGATCCTGGAGGCTTTA, n1025, 5’CAGGATAGGACGAGTGTG. The obtained PCR products were denatured and run on 10% polyacrylamide gels for 14 to 16 hours at room temperature and at 4°C, respectively. After the run, the bands were visualized using a standard silver-staining protocol. Polymerase chain reaction products showing aberrant patterns were amplified again prior to direct sequencing with an ABI 377 sequencer (Applied Biosystems, Foster City, Calif). For verification of the mutation and screening of the control sample, we developed a restriction fragment length assay using primers n1005 (5’AGCTGACACTTCTCCATCTCACG) and Z n1070 (5’TAGAGGTATGTAGGAATCTCTCT) and n1066 (5’TGGACAGATCATCTGPAGGGTTA, n1025, 5’CAGGATAGGACGACATGAGTGT. Annealing temperatures were optimal at 65°C, and human brain RNA (Clontech, Palo Alto, Calif) was successfully used as template. Singleand nested-PCR attempts, however, failed to amplify the GABRG2 cDNA from total RNA derived from leukocytes. For the prediction of possible cryptic splice–donor sites within intron 6, the 1998 version of an online splice site predictor program was used (www.fruitfly.org).

A FREQUENT POLYMORPHISM IN THE GABRG2 GENE IS NOT ASSOCIATED WITHiae

To further analyze the possible role of the GABRG2 gene in epileptogenesis, we determined the genotype and allele frequencies of the exon 5 C588T polymorphism in the entire sample of 135 IAE patients as well as in 154 controls. Power calculation showed that the employed sample should provide a statistical power of 93.3% to detect a susceptibility factor with a genotypic relative risk of 2.50, assuming a type I error rate of 5% and a prevalence of the risk factor (T-allele) of 30% (SAS version 1988). The genotype distribution did not deviate significantly from that expected according to the Hardy-Weinberg equilibrium. The allele frequencies (χ² = 0.47; P = .49) and genotype frequencies (χ² = 2.09; P = .35) did not differ significantly between patients with IAE and controls (Table).

COMMENT

The IVS6+2T→G mutation found in family 510 destroys the 5’-splice site of intron 6, thus preventing the correct cleavage and removal of the intervening sequences from the pre–messenger RNA (mRNA). Since it was not possible to amplify the GABRG2 cDNA from peripheral blood cell templates, the effect of the mutation could not be analyzed directly. However, based on previous studies concerned with splice–donor site mutations (for example, 20-22) some predictions can be made. Most splice–donor site mutations lead to (1) exon skipping, (2) use of cryptic splice sites within the downstream intron, or, if the intron is small, to (3) intron inclusion.10 Because of the size of the GABRG2 intron 6 (approximately 38 kb), the latter is very unlikely. Alternatively, the IVS6+2T→G mutation could lead to the use of 1 of the 2 strong cryptic splice

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The predicted protein coded by this aberration stop codon at the joining site of exons 5 and 7 would lead to an RNA containing an aberrant spliced RNA would be truncated upstream from the first transmembrane domain and would therefore be predicted to be nonfunctional. Thus, we propose that the GABRG2 splice–donor site mutation reported here leads to a nonfunctional allele, which is likely to be the primary cause for epileptic seizures in this family.

The findings reported here suggest that a novel splice mutation of the GABRG2 gene causes a nonfunctional truncation of the GABAA receptor γ-subunit, contributing a major susceptibility effect to the pathogenesis of CAE and FC in a single family. Together with 2 previously identified amino acid exchanges,12,13 this truncation mutation extends the spectrum of GABRG2 mutations that confers monogenic effects to the pathogenesis of FC and CAE. Because the screening of 135 patients with IAE did not reveal more than 1 functional mutation and because of the negative results obtained in the association study, it is obvious that GABRG2 is not playing a major role in the pathogenesis of common IAE subtypes.

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<table>
<thead>
<tr>
<th>Sample with IAE (n = 135)</th>
<th>T/T</th>
<th>C/T</th>
<th>C/C</th>
<th>f(T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>0.037</td>
<td>0.348</td>
<td>0.615</td>
<td>0.211</td>
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<td>Controls (n = 154)</td>
<td>0.052</td>
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*(f(T) indicates frequency of T-allele; IAE, idiopathic absence epilepsies.)*

The probable effect of the IVS6+2T→G mutation on GABRG2 structure. A, Exon 6 and the adjacent part of intron 6, including an in-frame stop codon and 2 strong cryptic splice–donor sites within the intron. B, Exon-intron structure of GABRG2. The intron 6–splice–donor site mutation is indicated by the arrow. Exons are not drawn according to size. **Figure 2.** Probable effect of the IVS6+2T→G on GABRG2 structure. A, Exon 6 and the adjacent part of intron 6, including an in-frame stop codon and 2 strong cryptic splice–donor sites within the intron. B, Exon-intron structure of GABRG2. The intron 6–splice–donor site mutation is indicated by the arrow. Exons are not drawn according to size. C, Schematic representation of the probable outcome of exon 6 skipping. The excision of introns 5 and 6 and exon 6 followed by the fusion of exons 5 and 7 would create an in-frame stop codon. bp indicates base pair; kb, kilobase.

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Corresponding author and reprints: Ortrud K. Steinlein, MD, Institute of Human Genetics, University Hospital Bonn, Wilhelmstr 31, D-53111 Bonn, Germany (e-mail: ortrud.steinlein@ukb.uni-bonn.de).

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