



FULL-LENGTH ORIGINAL RESEARCH

Bilateral frontoparietal polymicrogyria, Lennox-Gastaut syndrome, and *GPR56* gene mutations

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SUMMARY

Purpose: Bilateral frontoparietal polymicrogyria (BFPP) has been reported in sporadic patients and in recessive pedigrees. Eleven mutations in *GPR56*, a gene encoding an evolutionarily dynamic G-protein-coupled receptor, have been identified in 29 patients from 18 families. The clinical features of BFPP include severe mental retardation, motor and language impairment, and epilepsy. No detailed description of the epilepsy is available for the patients reported to date. We report three consanguineous families in which four affected individuals with BFPP and *GPR56* mutations had Lennox-Gastaut syndrome.

Methods: Family studies, brain magnetic resonance imaging (MRI), electroencephalography (EEG)-video recordings, and mutation analysis.

Results: In Family 1, with one affected proband, we found an R565W change in the second extracellular loop of *GPR56*, involving a highly conserved aminoacidic residue. In family 2, with one

affected proband, we found an R79X change affecting the protein N-terminus and predicted to cause a premature truncation with loss of the G-protein-coupled receptor proteolytic site. In family 3, with two affected siblings, we found an R33P substitution in the protein N-terminus, involving a highly conserved aminoacidic residue. Epilepsy, present in all four patients, had started between ages 1 and 8 years, with infantile spasms in one patient and with *de novo* Lennox-Gastaut syndrome in the remaining three. All patients had Lennox-Gastaut syndrome when last observed, at ages 13 to 32 years.

Discussion: Several genes, when mutated, can cause malformations of cortical development that have been associated with the Lennox-Gastaut syndrome. BFPP caused by *GPR56* mutations represents an additional, although rare, genetically determined cause Lennox-Gastaut syndrome.

KEY WORDS: Polymicrogyria, Epilepsy, Lennox-Gastaut, *GPR56*.

Polymicrogyria is a developmental disorder characterized by multiple small and partly fused cortical gyri, with abnormal lamination (Harding & Copp, 1997; Barkovich et al., 2005). The spectrum of associated clinical manifestations ranges from normal

individuals with only selective impairment of cognitive function (Galaburda et al., 1985) to patients with severe encephalopathies and intractable epilepsy (Guerrini et al., 1992a).

Several polymicrogyria syndromes have been described that are characterized by different patterns of lobar expression (Barkovich et al., 1999), suggesting mutations of regionally expressed developmental genes. The most frequently observed syndromes of regional polymicrogyria include bilateral perisylvian polymicrogyria (Guerrini et al., 1992b; Kuzniecky et al., 1993), bilateral frontal or frontoparietal polymicrogyria

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(Guerrini et al., 2000; Piao et al., 2002, 2004), bilateral parasagittal parieto-occipital polymicrogyria (Guerrini et al., 1997), and unilateral multilobar polymicrogyria (Guerrini et al., 1998; Bartolomei et al., 1999).

Bilateral perisylvian polymicrogyria is genetically heterogeneous (Guerreiro et al., 2000) but is mainly X-linked, with a few families mapping to chromosome Xq28 (Villard et al., 2002). Its clinical expression includes mild cognitive impairment and pseudobulbar palsy in almost all patients, and severe symptomatic generalized epilepsy of the Lennox-Gastaut type in most (Guerrini et al., 1992a; Kuzniecky et al., 1993). Bilateral parasagittal parieto-occipital polymicrogyria has been reported in only sporadic patients, all having a homogeneous clinical syndrome associating mild cognitive impairment and complex partial seizures of occipitotemporal origin (Guerrini et al., 1998). Unilateral multilobar polymicrogyria is, with few exceptions, sporadic (Bartolomei et al., 1999; Chang et al., 2006), and is often associated with mild cognitive impairment, hemiparesis, and an age-related syndrome of epilepsy with sleep-related electrical status epilepticus and cognitive deterioration (Guerrini et al., 1998; Caraballo et al., 1999).

Bilateral frontal and frontoparietal polymicrogyria (BFPP) has been reported in sporadic patients and in recessive pedigrees (Guerrini et al., 2000; Piao et al., 2002, 2005). Linkage analysis in consanguineous families localized the responsible gene on chromosome 16q12.2–21 (Piao et al., 2002; Chang et al., 2003). Eleven independent mutations in *GPR56*, a gene encoding an evolutionarily dynamic G-protein-coupled receptor (GPCR), were identified in 29 patients from

18 families (Piao et al., 2004, 2005). The main clinical features of BFPP include severe mental retardation, developmental delay, gait and language impairment, and epilepsy of variable severity. Focal seizures and atypical absences were the main seizure types in two patient series (Piao et al., 2002; Chang et al., 2003).

We report three families in which four affected individuals with BFPP and *GPR56* mutations had Lennox-Gastaut syndrome, possibly indicating a close association between abnormal cortical development of the anterior brain and the electroclinical spectrum of symptomatic generalized epilepsy.

PATIENTS AND METHODS

As part of a research study that aims at establishing correlations between developmental abnormalities of the cerebral cortex and associated clinical features and, whenever possible, between genotype and phenotype, we were referred three consecutive probands with bilateral frontoparietal polymicrogyria (Figs 1A–C and 2). One proband (patient 1) had been reported briefly by Piao et al. (2004). Through family studies we identified a fourth affected individual (Figs 1D and 2). All four patients had Lennox-Gastaut syndrome and their clinical histories are described in detail in the following text. We performed mutation analysis of the *GPR56* gene in the three probands and extended the genetic study to available family members (Fig. 2). Informed consent was obtained from their respective human subject institutional review boards. Clinical data were obtained from referring physicians. Neuroimaging was performed using a 1.5T magnetic resonance imaging

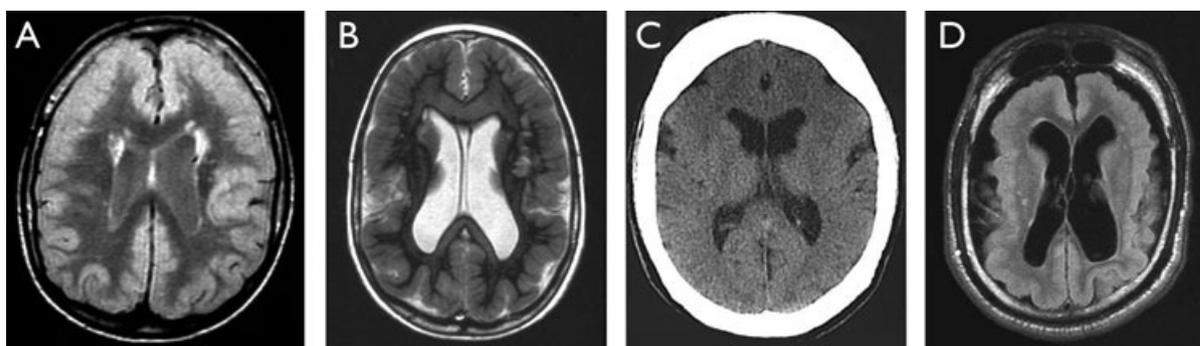
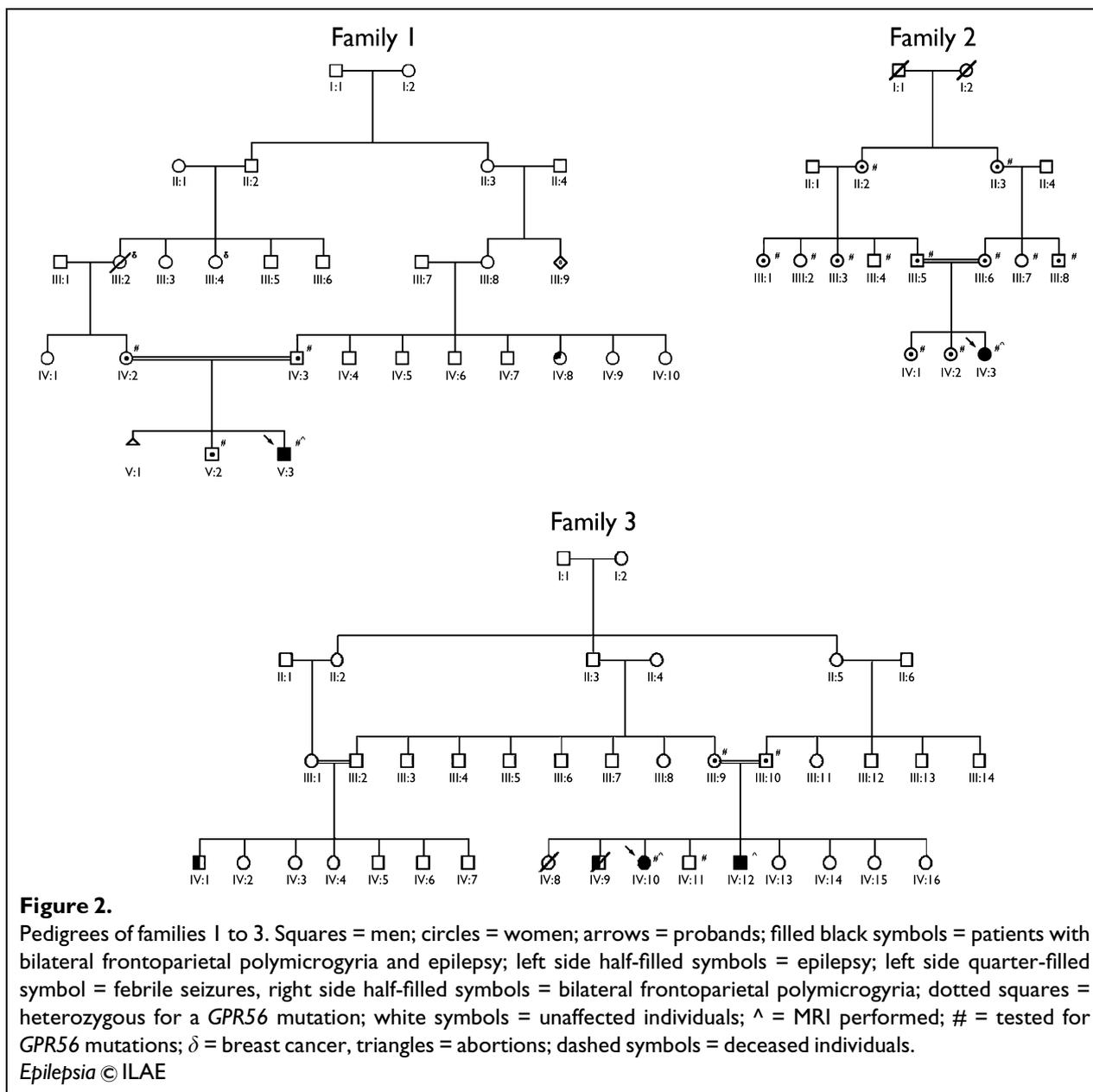


Figure 1.

6 (A) PD-weighted brain magnetic resonance imaging (MRI) scan of patient 1; (B) T2-weighted brain MRI scan of patient 2; (C) Computed tomography (CT) scan of the brain of patient 3; (D) T1-weighted brain MRI scan of patient 4. In all four patients neuroimaging shows irregular thickening of the cortex in the frontal and parietal lobes, with a simplified gyral pattern. Patients 2 and 4 also exhibit ventricular dilation, and the MRI scans in patients 1, 2, and 4 show patchy areas of increased signal abnormality in the periventricular or subcortical white matter.

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(MRI) system in three patients (Fig. 1A,B,D) and a computed tomography (CT) scanner in one patient (Fig. 1C).

Mutation analysis

Family members in whom mutation analysis was performed are indicated in Fig. 2. DNA was extracted from peripheral blood leukocytes using a DNA isolation Kit (DNAzol; MRC, Cincinnati, OH, USA) according to the manufacturer's protocol. *GPR56* coding regions and exon-intron boundaries, including about 50 base pairs upstream and downstream of the exons (GenBank accession number AF106858), were amplified by

polymerase chain reaction (PCR). Primers are available on request.

Heteroduplex analysis was performed using an automated denaturing high-performance liquid chromatography (DHPLC) instrument (WAVE DNA Fragment Analysis System; Transgenomic, La Jolla, CA, USA). Sample preparation for DHPLC analysis was carried out by denaturing and reannealing of unpurified PCR products. The temperature conditions required for the successful resolution of heteroduplexes were obtained using Navigator Software (Transgenomic). PCR conditions and DHPLC analysis temperatures are available on request. We mixed PCR products obtained from the patients with

amplicons from an unaffected individual. PCR products that showed an alteration in the DHPLC elution profile were purified using the GenElute PCR clean-up kit (Sigma Aldrich, St. Louis, MO, USA), cycle sequenced on both strands using the BigDye Terminator v 1.1 chemistry (Applied Biosystems, Foster City, CA, USA) and run on an ABI PRISM 3100-*Avant* genetic analyzer (Applied Biosystems). The mutations were described at nucleotide level according to the *GPR56* cDNA sequence (GenBank AF106858), in which the nucleotide +1 is the A of the ATG-translation initiation codon.

The nucleotide changes we observed were not found in 250 control DNA samples of mixed ethnic origin tested by DHPLC in our laboratory.

We analyzed the sequence of *GPR56* domains using the ClustalW Program (<http://www.ebi.ac.uk/clustalw/>), which is a compilation of multiple sequence alignments, representing protein domains that are conserved in molecular evolution.

RESULTS

Clinical and electroencephalography (EEG) findings of the four patients are summarized in Table 1.

Patient 1

Clinical and neuroimaging findings

The proband (1-V:3) was a 19-year-old boy who was born to first-cousin Italian parents. Pregnancy and delivery were uneventful. Delayed milestones were noticed during

the second year of life. Severe mental retardation with spastic quadriplegia became progressively apparent. On neurologic examination at age 19 years, he presented exotropia, wide-based gait, brisk deep-tendon reflexes, and bilateral ankle clonus. Speech was limited to a few isolated words. Head circumference was 54 cm (<50th percentile).

A generalized clonic seizure during fever occurred at age 1 year. Atypical absences and tonic seizures started at age 2 years and proved intractable. Multiple per-day atonic and generalized tonic-clonic seizures appeared subsequently. Since age 8 years, he experienced several episodes of nonconvulsive status epilepticus. Intractable generalized seizures were resistant to multiple anti-epileptic drugs and had not changed their characteristics when the patient was last seen. Interictal EEG showed bursts of generalized slow spike-and-wave complexes and, during slow-wave sleep, bursts of multiple spikes (Fig. 3, left), and generalized multiple spike-and-wave complexes with frontal predominance. During EEG monitoring, numerous sleep-related tonic seizures were captured (Fig. 4, left). Brain MRI (Fig. 1A) showed BFPP.

Mutation analysis

In patient 1-V:3, DHPLC analysis showed an altered elution profile in exon 12. Sequence analysis revealed a c.1693C>T substitution (Fig. 5A), leading to a R565W change in the second extracellular loop of *GPR56* (Fig. 6). In both parents (individuals 1-IV:2, 1-IV:3), sequence analysis of exon 12 revealed heterozygosity for the R565W change, in agreement with the autosomal recessive inheritance.

Table 1. Main clinical features in four patients with *GPR56* mutations

Patient	Age (years)	Age at diagnosis of developmental delay (years)	Age at seizure onset (years)	Seizure types	Interictal EEG	Neurologic examination
1	19	1.5	1	Tonic, atonic, generalized tonic-clonic, atypical absences; recurrent nonconvulsive status epilepticus	Generalized bursts of multiple spikes; slow SW and poly SW complexes	Severe mental retardation, exotropia, spastic quadriplegia, wide-based gait, brisk deep-tendon reflexes, bilateral ankle clonus, poor language skills
2	13	1	2	Infantile spasms; tonic and atonic seizures	Generalized bursts of multiple spikes; slow SW complexes	Severe mental retardation, mild spasticity, brisk deep-tendon reflexes, wide-based gait, exotropia and nystagmus on vertical endgaze, poor language skills
3	27	1.5	5	Atypical absences, generalized tonic-clonic, tonic	Generalized bursts of multiple spikes; slow SW complexes	Severe mental retardation, unsteady gait, brisk deep tendon reflexes, strabismus, poor language skills
4	32	1.5	8	Tonic, atypical absences; recurrent non-convulsive status epilepticus	Slow SW complexes; multifocal spikes	Severe mental retardation, exotropia, pendular nystagmus, brisk deep tendon reflexes, wide-based gait, poor language skills

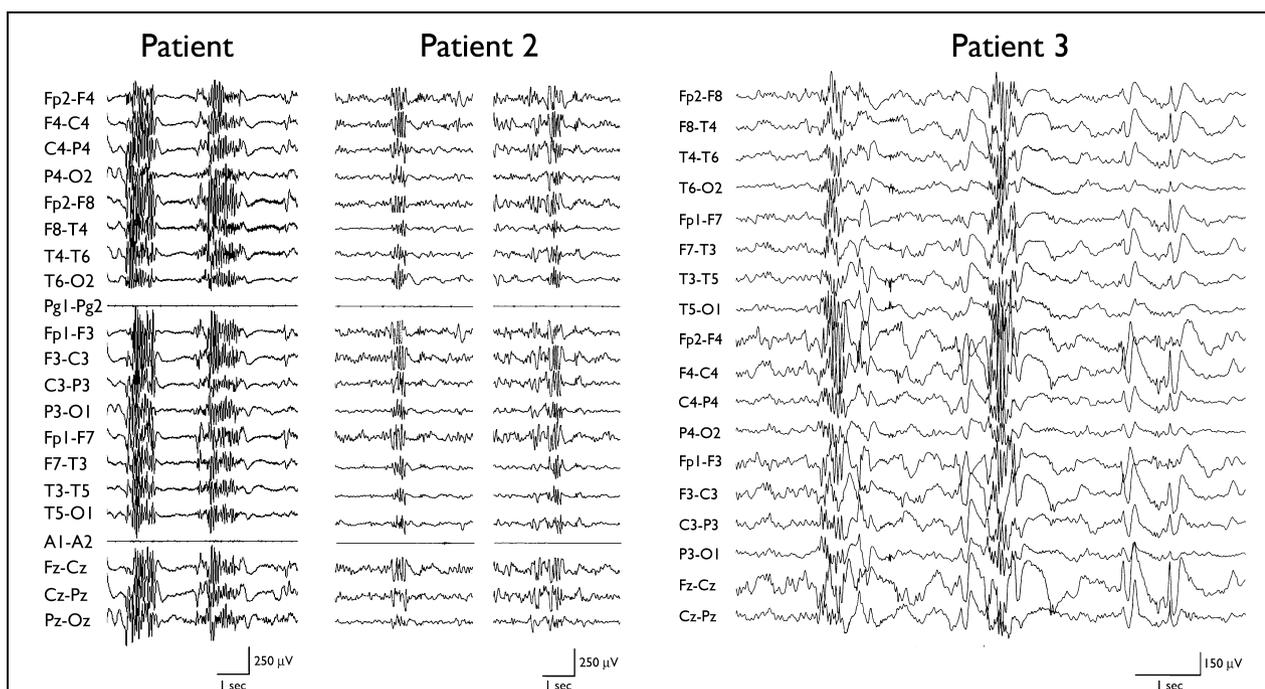


Figure 3.

Left: patient 1; middle: patient 2; right: patient 3. Generalized bursts of multiple spikes and slow spike and wave discharges with anterior predominance are seen during sleep.

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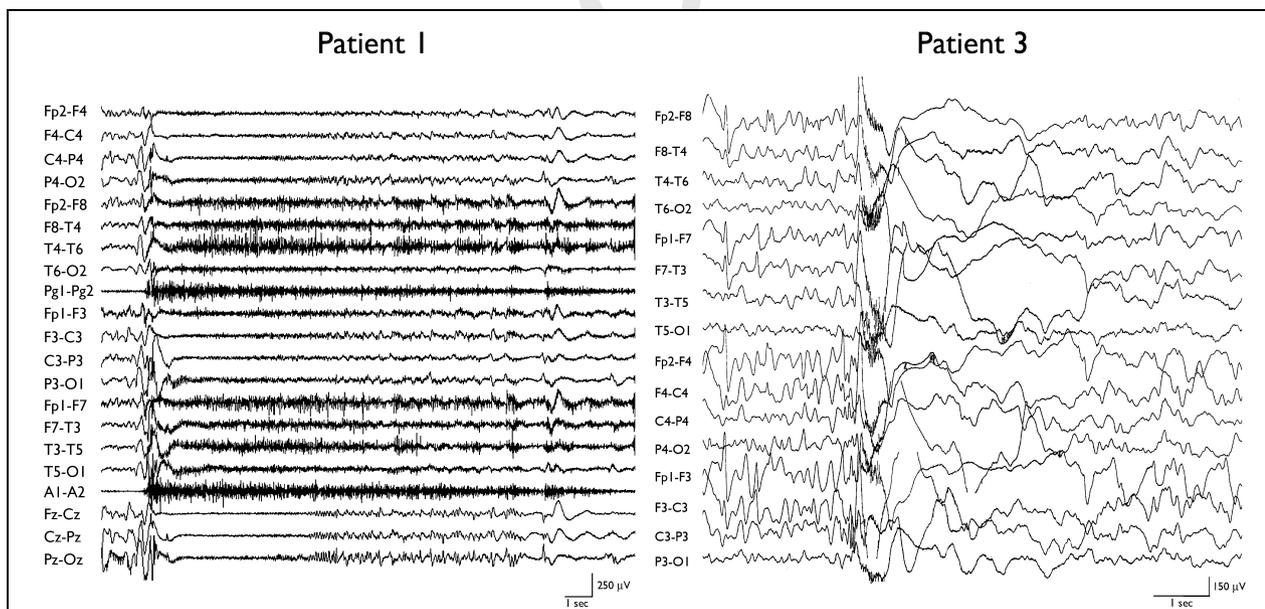


Figure 4.

Left: Patient 1. A generalized tonic seizure is recorded during sleep. A burst of generalized polyspikes is followed by an electrodecremental event progressively translating into low voltage fast activity. A tonic contraction is visible in the deltoid muscles, lasting about 15 s. Pg1-Pg2 = left deltoid; A1-A2 = right deltoid. Right: Patient 3. Interictal slow spike and wave activity over the anterior brain regions is replaced by a generalized electrodecremental event lasting about 5 s and accompanied by a tonic seizure.

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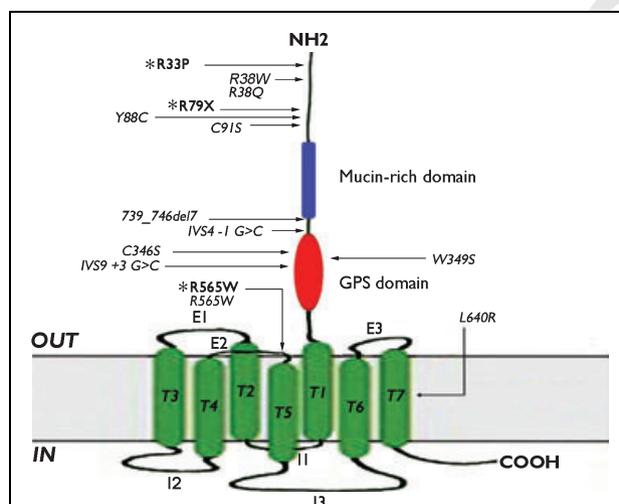
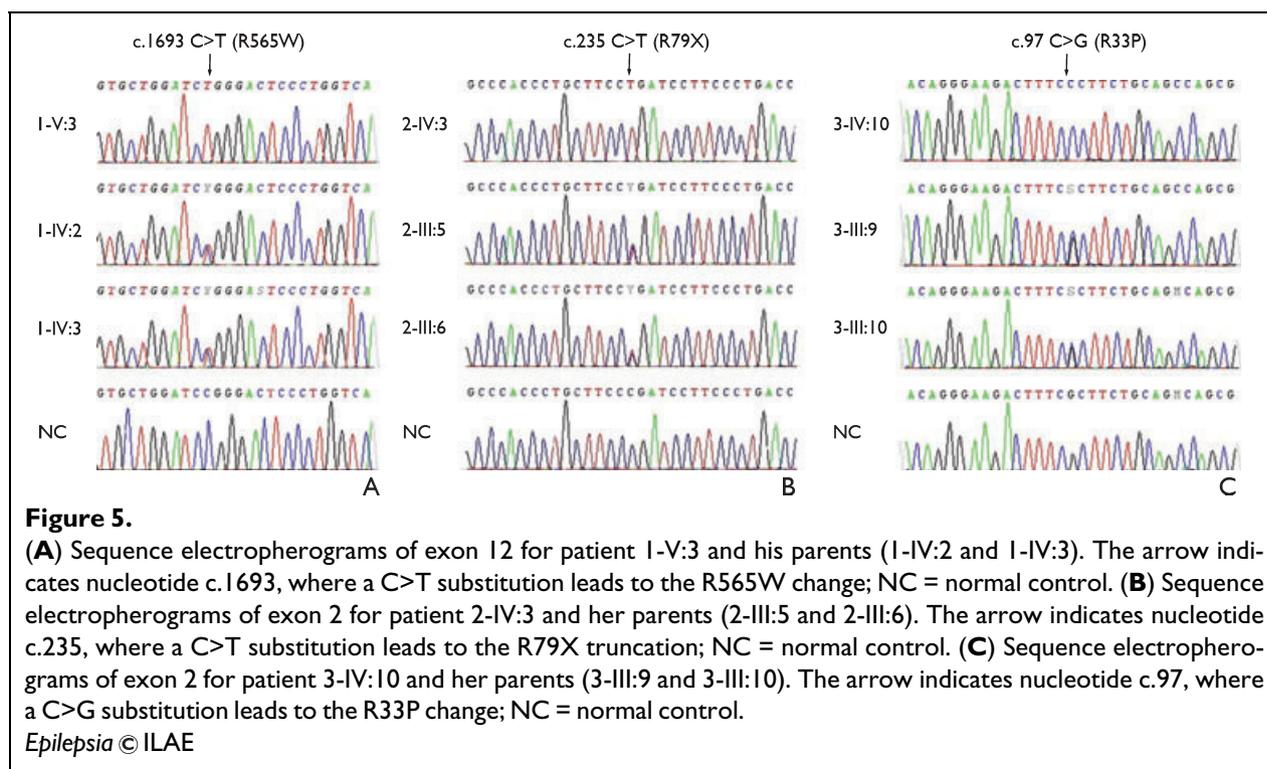


Figure 6.
 Schematic representation of the GPR56 protein. Previously described mutations are indicated in italics (Piao et al., 2004, 2005). Asterisks indicate the mutations identified in our patients: two missense mutations, one at the tip of the N-terminus (R33P), one in the second extracellular loop (R565W), and one nonsense mutation at the tip of the N-terminus (R79X). I = intracellular loop; E = extracellular loop; T = transmembrane domain.

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Patient 2

Clinical and neuroimaging findings

The proband (2-IV:3), a 13-year-old girl, was born to first-cousin Italian parents. Severe developmental delay was apparent within the first years of life. Autonomous walking was reached at 5 years. Neurologic examination revealed mild spasticity, brisk deep-tendon reflexes, wide-based gait, exotropia, and nystagmus on vertical end gaze. Speech was limited to a few isolated words.

A generalized clonic seizure during fever occurred at age 2 years. Since age 2 years 6 months, infantile spasms appeared that proved resistant to drugs. At around age 4 years, the spasms progressively acquired the characteristics of tonic seizures, and tonic seizures also appeared, both causing drop attacks. Tonic seizures were also intractable and did not change their characteristics over the following years. Interictal EEGs showed bursts of generalized multiple spikes and spike-wave discharges during sleep (Fig. 3, middle part). Brain MRI (Fig. 1B) showed bilateral frontoparietal polymicrogyria.

Mutation analysis

In patient 2-IV:3, DHPLC analysis revealed an altered elution profile in exon 2. Sequence analysis revealed a c.235C>T substitution on both alleles (Fig. 5B), leading to a Arg79STOP (R79X) change in the extracellular N-terminus of the protein (Fig. 6). This substitution causes a

truncation of the protein. Parents of both probands (individuals 2-III:5 and 2-III:6) were heterozygous for the same nonsense mutation.

Patients 3 and 4

Clinical and neuroimaging findings

The proband (patient 3; 3-IV:10) was a 27-year-old woman, born to first-degree cousin Turkish parents. A proband's brother (3-IV:9) had died at age one year from complications of status epilepticus in the context of a syndrome of early onset intractable seizures and severe delay. A maternal first cousin (3-IV:1) had mental retardation and epilepsy.

Developmental delay was diagnosed during the second year of life. Generalized seizures appeared at age 5 years, rapidly acquiring the characteristics of Lennox-Gastaut syndrome, and persisting to the time of this study. Atypical absences, tonic and tonic-clonic attacks, and generalized interictal slow spike-and wave discharges were recorded during EEG monitoring (Fig. 3, right and Fig. 4, right). Neurologic examination revealed severe mental retardation, unsteady gait with brisk deep tendon reflexes, and strabismus. Speech was limited to a few isolated words. Brain CT scan showed cortical thickening and a simplified gyral pattern in the frontoparietal regions (Fig. 1C), consistent with polymicrogyria.

The proband's 32-year-old affected brother (patient 4; 3-IV:12) was severely mentally retarded. He was diagnosed as being developmentally delayed since the second year of life. Seizures started when he was 8 years old, rapidly exhibiting the features of Lennox-Gastaut syndrome as atypical absence and generalized tonic attacks. Several episodes of nonconvulsive status epilepticus were noticed for the first time when the patient was 30 years old and were then brought under control. Interictal EEG showed generalized and multifocal spikes and slow-wave activity. His brain MRI scan showed bilateral BFPP (Fig. 1D).

Mutation analysis

DHPLC analysis in patient 3-IV:10 and in her affected brother (3-IV:12) resulted in an altered elution profile in exon 2. Subsequent sequencing showed a c.97C>G substitution (Fig. 5C), resulting in an Arg33Phe (R33P) change in the extracellular N-terminus of *GPR56* (Fig. 6). Sequence analysis of exon 2 performed on both of the proband's parents (individuals 3-III:9 and 3-III:10) showed that they were heterozygous for the R33P change (Fig. 5C).

DISCUSSION

G-Protein-coupled receptors (GPCRs) protein family

GPR56 is a member of the GPCR family. GPCRs, which represent the largest receptor family in the human genome, are involved in the recognition and transduction

of such messages as light, odorants, small molecules including amino-acid residues, and nucleotides and peptides, as well as proteins (Liu et al., 1999). GPCRs control the activity of enzymes, ion channels, and transport of vesicles via the catalysis of the GDP-GTP exchange on heterotrimeric G proteins (Ga-bg) (Bockaert & Pin, 1999). GPCRs have in common a central core domain composed of seven transmembrane helices (TM-I through TM-VII) connected by three intracellular and three extracellular loops (Baldwin, 1993). Two highly conserved cysteine residues form a disulfide link that is probably important for the packing and stabilization of a restricted number of conformations of these seven transmembrane helices (Bockaert & Pin, 1999).

The GPR56 protein itself

GPR56 is a member of the family B GPCRs (secretin-like receptors), in which the relatively long N-terminal domain and the extracellular loops play a role in the binding of molecular weight hormones such as glucagon, secretin, and α -latrotoxin. The N-terminal binding unit contains four cysteines that probably form disulfide bridges and mucin-like domains (cysteine box, also named GPS, G-protein-coupled receptor proteolytic site) (Liu et al., 1999; Fredriksson et al., 2003). The N-terminal domain shows only 64% of homology between mouse and humans, versus 80% of homology over the rest of the protein. Normally, the GPR56 protein undergoes two major modifications: GPS domain-mediated protein cleavage and N-glycosylation; after these modifications the N-terminal fragment can be released from the cell surface (Jin et al., 2007). Mutations affecting the GPS domain make GPR56 resistant to cleavage and impair its trafficking, thereby preventing its expression at the cell surface (Jin et al., 2007). Mutations affecting the N-glycosylation sites allow GPR56 to be proteolytically processed, thereby reducing its expression at the cell surface (Jin et al., 2007). GPR56 is expressed in neuronal progenitor cells during neurogenesis in the ventricular and subventricular germinal zones. Its deficient expression causes abnormal development of the frontoparietal neocortex (Piao et al., 2004), making it likely that GPR56 regulates cortical development of these regions (Piao et al., 2004).

GPR56 gene mutations in our patients

The mutations observed in our patients are located in the extracellular domain and affect neither the cysteine box domain, which serves as a cleavage site, nor the seven N-glycosylation sites.

In family 1, the R565W change in the second extracellular loop of the protein (Fig. 6) involves an amino acid residue that is highly conserved across a wide range of vertebrate G-protein-coupled receptors. This mutation had previously been also identified in a consanguineous Bedouin family (Piao et al., 2004) with three affected

children. Functional studies demonstrated that the R565W mutation did not cause severe defects in protein trafficking or cell surface expression (Jin et al., 2007). In family 2, the R79X change is located in the early portion of the protein (Fig. 6), and is predicted to cause a premature truncation with loss of the GPS domain and consequent severe loss of function. Absence of this GPS domain can lead to the loss of the proteolytic activity of GPR56 and of its expression at the cell surface. In family 3, the R33P substitution in the extracellular N-terminus (Fig. 6) involves an aminoacidic residue that is highly conserved across a wide range of vertebrate G-protein-coupled receptors. It has been demonstrated that missense mutations at the tip of the N-terminal domain (ex: R338Q or R38W) produce proteins with reduced intracellular trafficking and poor cell surface expression (Jin et al., 2007). We may hypothesize that the R33P mutation has a similar effect on the protein.

Our observations

Clinical and EEG studies of the three families reported herein indicate that *GPR56* mutations cause a severe encephalopathy in which bilateral polymicrogyria of the frontoparietal cortex and patchy bilateral high signal abnormalities in the white matter are associated with the electroclinical features of the Lennox-Gastaut syndrome. Epilepsy started between the ages of 1 and 8 years, exhibiting the typical characteristics of the syndrome from the onset in three patients and following initial infantile spasms in one. We found no major clinical or anatomic differences in severity in the four affected patients reported herein. However, subtle differences in developmental skills, which would have been expected from the different functional consequences of the R33P and R79X mutations (N-terminal) and the R565W (extracellular domain), might have been obscured by the severe epileptic encephalopathy. No detailed description of the epilepsy type was available for most of the previously reported patients with BFPP, but atonic drops, atypical absences, and generalized myoclonus were frequently reported in those whose clinical information could be collected (Piao et al., 2005).

Polymicrogyria and epilepsy

The term polymicrogyria defines an excessive number of abnormally small gyri that produce an irregular cortical surface (Barkovich et al., 2005). Polymicrogyria is a common cortical malformation and is associated with a wide spectrum of anatomic patterns and clinical syndromes. Brain pathology demonstrates abnormal development or loss of neurons in middle and deep cortical layers (Englund et al., 2005), variably associated with an unlayered cortical structure. To date, polymicrogyria has been associated with mutations of only a few genes including *SRPX2* (Roll et al., 2006), *PAX6* (Glaser et al., 1994), *TBR2* (Baala et al., 2007), *KIAA1279* (Brooks et al.,

2005), *RAB3GAP1* (Aligianis et al., 2005), and *COL18A1* (Sertié et al., 2000).

There are no anatomopathologic studies that have characterized the pattern of cortical laminar alterations in patients with *GPR56* gene mutations, but it has been suggested that the imaging characteristics of BFPP, including myelination defects and cerebellar cortical dysplasia, are reminiscent of those of the so-called cobblestone malformations (muscle-eye-brain disease and Fukuyama congenital muscular dystrophy) that are also associated with N-glycosylation defects in the developing brain (Guerrini et al., 2008). Such malformations feature a spectrum of cytoarchitectural abnormalities including unlayered polymicrogyria, abnormal cell morphology and alignment, and cortical vascular patterns (Takada et al., 1988).

Numerous observations have linked polymicrogyria with a spectrum of epilepsy phenotypes. Epileptogenicity of polymicrogyria and its mechanisms are not known, but a considerable number of patients do not have epilepsy (Guerrini & Filippi, 2005). Experimental models produced by localized freezing suggest widespread functional disruption, with downregulation of different gamma-aminobutyric acid (GABA) receptor subunits extending far beyond the visualized abnormality (Redecker et al., 2000). Diffuse epileptogenesis is frequently encountered, even with seemingly limited abnormalities (Guerrini et al., 1992b). Intracranial recordings suggest large epileptogenic networks that extend well beyond the limits of the visible abnormality (Guerrini et al., 1992b; Chassoux et al., 2008). Epilepsy related to polymicrogyria has variable severity, including cases with good outcome and spontaneous remissions, even after a period of intractability (Guerrini et al., 1998). Surgical treatment of epilepsy is applicable to a very limited number of patients in whom large resections are feasible (Chassoux et al., 2008).

Lennox-Gastaut syndrome has multiple etiologies, and although it can appear as a “cryptogenic” disorder, in the absence of any detectable brain abnormality, it is most often associated with focal, multifocal, or diffuse brain damage (Genton et al., 2000; Guerrini, 2006). Gloor et al. (1968) stressed how the association of diffuse cortical and subcortical damage was particularly frequent in patients with the slow spike and wave discharge trait. The observations reported herein and those of previous studies point to a propensity for multilobar polymicrogyria involving the frontoparietal regions to generate diffuse slow spike and wave patterns. For example Lennox-Gastaut syndrome was observed in most patients with bilateral perisylvian and central polymicrogyria (Guerrini et al., 1992a; Kuzniecky et al., 1993, 1994). Epilepsy with sleep-related electrical status epilepticus (or continuous spike and waves during slow sleep) has been reported in patients with multilobar polymicrogyria also involving the frontal lobes, unilaterally or bilaterally (Guerrini et al., 1998; Caraballo et al., 1999). There are no reports of epilepsies

with diffuse slow-spike and wave in patients with parieto-occipital polymicrogyria (Guerrini et al., 1997) or in those with focal cortical dysplasia or other forms of regional developmental cortical abnormality.

CONCLUSIONS

Lennox-Gastaut syndrome can be cryptogenic or symptomatic. Symptomatic forms have been associated with multiple etiologies, and abnormal cortical development is frequently encountered (Genton et al., 2000). Several genes, when mutated, can cause malformations of cortical development that have been frequently associated with the Lennox-Gastaut syndrome, including the *LIS1*, *DCX*, *TSC1*, and *TSC2* genes (Guerrini & Carrozzo, 2001; Guerrini & Filippi, 2005). BFPP caused by *GPR56* mutations represents an additional, genetically determined malformation of cortical development that can cause Lennox-Gastaut syndrome.

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We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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