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FULL-LENGTH ORIGINAL RESEARCH

Low penetrance and effect on protein secretion of *LGI1* mutations causing autosomal dominant lateral temporal epilepsy

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Summary

Purpose: To describe the clinical and genetic findings of four families with autosomal dominant lateral temporal epilepsy.

Methods: A personal and family history was obtained from each affected and unaffected subject along with a physical and neurologic examination. Routine electroencephalography and magnetic resonance imaging (MRI) studies were performed in almost all patients. DNAs from family members were screened for *LGI1* mutations. The effects of mutations on Lgi1 protein secretion were determined in transfected culture cells.

Key Findings: The four families included a total of 11 patients (two deceased), six of whom had lateral temporal epilepsy with auditory aura. Age at onset was in the second decade of life; seizures were well controlled by antiepileptic treatment and MRI studies were normal. We found two pathogenic *LGI1* mutations with uncommonly low penetrance: the *R136W* mutation, previously detected in a sporadic case with telephone-induced partial seizures, gave rise to the epileptic phenotype in three of nine mutation carriers in one family; the novel *C179R* mutation caused epilepsy in an isolated patient from a family where the mutation segregated. Another novel pathogenic mutation, *I122T*, and a nonsynonymous variant, *I359V*, were found in the two other families. Protein secretion tests showed that the *R136W* and *I122T* mutations inhibited secretion of the mutant proteins, whereas *I359V* had no effect on protein secretion; *C179R* was not tested, because of its predictable effect on protein folding.

Significance: These findings suggest that some *LGI1* mutations may have a weak penetrance in families with complex inheritance pattern, or isolated patients, and that the protein secretion test, together with other predictive criteria, may help recognize pathogenic *LGI1* mutations.

KEY WORDS: Autosomal dominant lateral temporal epilepsy, LGI1, Mutation, Low penetrance, Protein secretion.

Autosomal dominant lateral temporal epilepsy (ADLTE; OMIM 600512), also named autosomal dominant partial epilepsy with auditory features (ADPEAF), is a well-defined condition characterized by onset in adolescence or early adulthood, lateral temporal seizures with prominent auditory or aphasic auras, normal MRI, and overall benign outcome (Ottman et al., 1995). Seizures in ADLTE/ADPEAF are sometimes triggered by sensory (usually acoustic) stimuli (Michelucci et al., 2003, 2004). Mutations associated with ADLTE/ADPEAF are found in the leucine rich, glioma inactivated 1 (*LGI1*) gene (Kalachikov et al., 2002; Morante-Redolat et al., 2002). To date, more than 25 *LGI1* mutations have been identified in families with a rather homogeneous phenotype and autosomal dominant inheritance pattern (Nobile et al., 2009; Heiman et al., 2010; Kawamata et al., 2010). Overall, *LGI1*mutations-account for about 50% of ADLTE/ADPEAF families (Michelucci et al., 2003; Ottman et al., 2004).

LGI1 does not encode an ion channel subunit. The structure of its protein product consists of an N-terminal domain composed of four leucine rich repeats (LRRs; Buchanan & Gay, 1996) and a C-terminal 7-repeat domain named EPTP (beta-propeller; Staub et al., 2002), both of which mediate

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protein–protein interactions. The Lgi1 protein is secreted by transfected culture cells and ADLTE-related mutations prevent secretion of mutant proteins, suggesting a loss of function effect of mutations (Senechal et al., 2005).

In this article, we describe four Italian ADLTE families exhibiting alterations in the *LGI1* sequence that result in amino acid substitutions. Our genetic and cell transfection findings show that three of these amino acid changes are disease-causing mutations, two of which have a peculiar low penetrance, whereas the fourth substitution turned out to be a rare nonpathogenic variant with no effect on protein secretion.

Patients and Methods

The four families are shown in Fig. 1. A personal and family history was obtained from each affected and unaffected member along with physical and neurologic examination. Routine electroencephalography (EEG) and magnetic resonance imaging (MRI) studies were performed in almost all patients. All subjects participating in the study gave written informed consent.

DNA was extracted from blood by standard methods and *LGI1* exons were polymerase chain reaction (PCR) amplified (conditions in Michelucci et al., 2003) and sequenced

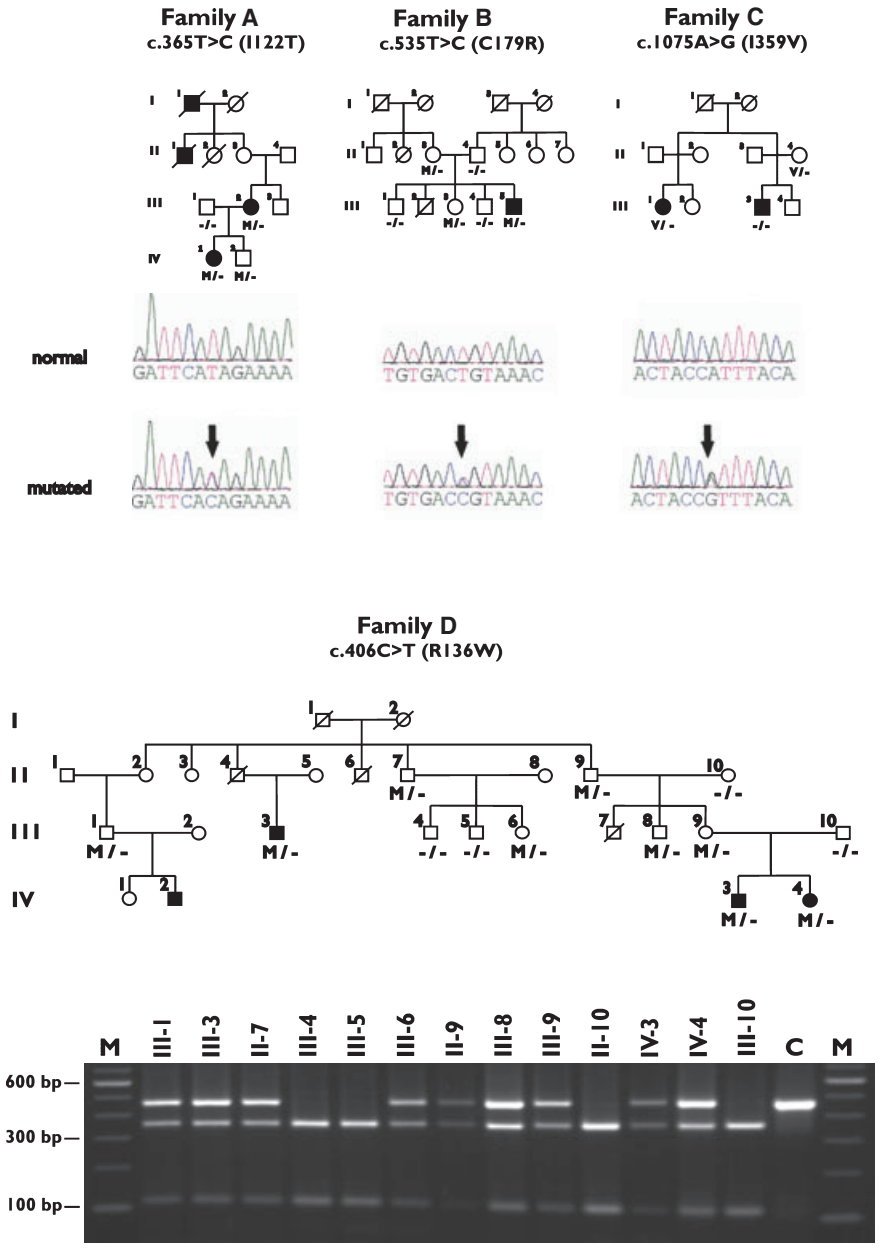

Figure 1.
Family pedigrees and segregation of mutations. Circles denote females, squares denote males, and blackened symbols denote subjects with epilepsy. The type of missense mutation is indicated for each family. Individuals carrying one mutant and one normal allele are denoted by M/−, those carrying the neutral variant c.1075A>G by V/−, and those with no mutations by −/−. Original sequence tracings used to detect disease alleles are shown below pedigrees A, B, and C. Mutated nucleotides are indicated by arrows. RFLP analysis of *LGI1* exon 4 PCR products from the indicated members of family D are shown below the pedigree. The mutation eliminates an MspI restriction site, giving rise to an undigested mutant allele. C, undigested control; bp, base pairs; M, molecular weight marker ladder.

with the Big Dye Terminator Cycle sequencing kit (ABI PRISM, Applied Biosystems, Carlsbad, CA, U.S.A.). The c.406C>T mutation, which eliminates an MspI restriction site in *LGI1* exon 4, was also revealed by restriction fragment length polymorphism (RFLP) analysis, as described (Michelucci et al., 2007). The other mutations were also revealed by other methods such as allele specific oligonucleotide (ASO) or denaturing high performance liquid chromatography (DHPLC) analysis.

Predictions of pathogenicity of *LGI1* mutations were made with Polymorphism Phenotyping (PolyPhen; http://genetics.bwh.harvard.edu/pph/) and Sorting Intolerant from Tolerant (SIFT; http://sift.jcvi.org/) programs, and using the Grantham matrix (Grantham, 1974).

Cell transfection assays were performed as described in detail previously (Furlan et al., 2006). Briefly, *LGI1* wild-type or mutant expression constructs containing a C-terminal Flag peptide in frame with the *LGI1* cDNA sequence were transfected into human embryonic kidney 293 (HEK293) cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.) and following the manufacturer's instructions. Twenty-four hours after transfection, cells were washed twice and incubated in serum-free medium for 16–20 h. Cells were then lysed and the medium was collected and concentrated about 20× using Centricon YM30 concentrators (Millipore, Billerica, MA, U.S.A.). Aliquots of cell lysates and concentrated medium were loaded on a sodium dodecil sulphate-polyacrilamide gel electrophoresis (SDS-PAGE) gel and analyzed by Western blot using the anti-Lgi1 antibody ab30868 (Abcam, Cambridge, U.K.) or the anti-Flag antibody F7425 (Sigma-Aldrich, St. Louis, MO, U.S.A.).

Results

Case description

The clinical, EEG, and neuroimaging findings of the patients from the four families studied are summarized in Table 1. Pedigrees are shown in Fig. 1.

Family A

The proband IV:1, a 30-year-old woman, started to experience seizures at 19 years. Over the following years, she experienced stereotyped daytime episodes of ''twitter in both ears, worse on the left side'' associated with sensation of dizziness and followed, rarely, by an impairment of consciousness. Only two secondarily generalized tonic seizures occurred (one after treatment was discontinued). Neurologic examination, brain MRI scan, and EEG studies were normal. She was first treated with phenobarbital, but it was treatment with lamotrigine at a daily dose of 200 mg that determined a partial seizure control (only sporadic episodes consisting of acoustic aura persisted). The medical history of the proband's mother (III:2) is characterized by two generalized tonic–clonic seizures, during which no aura was reported. Both EEG and MRI scan were normal. She is currently taking antiepileptic drugs (phenobarbital) with complete seizure control. The grandmother's brother II:1 died at 70 years; a detailed clinical history is lacking but sporadic tonic–clonic seizures during sleep were reported. Patient I:1, who died at 80 years, also experienced in his life sporadic tonic–clonic seizures but clinical details are not available.

Family B

The proband III:5, a 35-year-old man, had normal psychomotor development and no history of febrile seizures. He experienced a first nocturnal tonic generalized seizure at 14 years and, a year later, episodes consisting of bilateral buzzing, a whistle (bilateral or in left ear), a loud noise followed by confusion, vertigo, and infrequent secondary generalization. Seizures were triggered by some noises (such as far noises, ''atypical whistle''). His neurologic examination and MRI scan were normal. He is currently on polytherapy with phenobarbital 150 mg/day and carbamazepine 1,200 mg/day with good control of seizures (sporadic acoustic simple partial seizures persist).

Family C

The proband III:1 is a 24-year-old woman. Her seizures began at 11 years and consisted of auditory auras described like ''little song or repetition of words or distortion of human voices with volume changes'' with slight disturbance of alertness. Rare secondarily generalized tonic seizures were also described. Seizures were triggered by specific auditory stimulations such as noise of elevator or airplane. Treatment with oxcarbazepine had partial effect, as seizures continued to occur at a monthly frequency. The drugs tried previously included valproate and topiramate. MRI scan and neurologic examination were normal. Various EEG studies always showed slow waves and epileptiform abnormalities on the right temporal lobe. Patient III:3, a 33 year-old man, had focal motor seizures preceded by short auditory aura reported as ''Pink Floyd's song'' or whistle in bilateral ears, worse on the right side; no secondary generalization or trigger were reported. Treatment with carbamazepine resulted in a good seizure control (only 3–4 episodes per year).

Family D

The proband IV-3, a 24-year-old man, had two focal secondarily generalized seizures preceded by a short auditory aura reported as ''a non-fine tuned radio noise.'' The patient was seizure free on valproate for 4 years, but had a seizure with overlapping features while decreasing the daily dose of the medicament. Wake and sleep EEGs showed rare left temporal sharp waves on a normal background activity. Computed tomography (CT)/MRI, mental level, and neurologic examination were normal. The proband's sister, IV-4, aged 22 years, had two seizures at age 10 years with eye deviation and secondary generalization during nocturnal

Table 1. Clinical, EEG, and neuroimaging findings in the patients with *LGI1* mutations

Family	Pt	Sex/age	Age of onset	Auditory features	Early signs	Late signs	Seizure frequency	EEG	MRI	Therapy
A[a]	I:1	M/81+	Unknown	No	No	Generalized tonic–clonic seizures	–	–	–	–
A[a]	II:1	M/70+	Unknown	No	No	Generalized tonic–clonic seizures	–	–	–	–
A	III:2	F/51	19	No	No	Generalized tonic–clonic seizures	Seizure-free	Normal	Normal	PB
A	IV:1	F/30	19	Twitter in both ears (worse on the left side)	Vertiginous and psychic symptoms	Impairment of consciousness and sporadic secondary generalization	Sporadic SPS	Normal	Normal	LTG
B	III:5	M/35	14	Bilateral buzzing, a whistle, a loud noise	Vertiginous and psychic symptoms	Confusion and aphasia. Sporadic secondary generalization	Sporadic SPS	Slow waves and spikes in the left temporal area	Normal	PB+CBZ
C	III:1	F/24	11	Little song or repetition of words or distortion human voices	Slight disturbance of alertness and psychic symptoms	Motor phenomena and sporadic secondary generalization	Monthly	Slow waves and epileptiform abnormalities in the right temporal lobe	Normal	OXC
C	III:3	F/33	18	Pink Floyd's song or whistle (worse on the right side)	No	Motor phenomena	Sporadic SPS	Normal	Normal	CBZ
D	III-3	M/45	Childhood	No	No	Generalized tonic–clonic seizures	Seizure-free	Normal	Normal	PB
D	IV:3	M/24	18	A non–fine tuned radio noise	No	Secondary generalization	Sporadic SPS	Rare left temporal sharp waves	Normal	VPA
D	IV:4	F/22	10	Whistle at the right ear <1 min	No	Secondary generalization	Sporadic SPS	Isolated temporal sharp waves	Normal	VPA

SPS, simple partial seizures; PB, phenobarbital; LTG, lamotrigine; CBZ, carbamazepine; OXC, oxcarbazepine; VPA, valproic acid.
[a]No further information available (patient died).

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sleep. Occasionally she also experienced auditory simple partial seizures (whistle at the right ear lasting about 1 min). Eight years later, valproate decrease coincided with a new tonic–clonic seizure during sleep. Awake and sleep EEG recordings showed isolated left temporal sharp waves on a normal background activity. MRI scans were normal. The proband's mother III-9, aged 52 years, never reported auditory and/or seizure disorders. Awake EEG recordings disclosed rare theta waves on both temporal leads. Subject III-3, a 45-year-old man, is affected by muscular dystrophy and childhood-onset generalized tonic–clonic epilepsy that is well controlled with phenobarbital. He never reported auditory symptoms. Patient IV-2, a 19-year-old man with normal mental functioning, was reported to have childhood-onset epilepsy. He refused to participate in the study.

Molecular genetic analysis

In family A, a heterozygous *LGI1 c.365T>C* nucleotide change was found in the proband IV-1 by direct sequencing of *LGI1* exons (Fig. 1; numbering from the first nucleotide of the start codon). This mutation occurs in exon 4 and results in a substitution of the isoleucine at position 122 with a threonine residue (*I122T*). The same mutation was found also in the affected mother III-2 and unaffected brother IV-2 but not in 130 unrelated healthy controls of Italian ancestry. The I122 is conserved in many species, including rat, mouse, chicken, and zebrafish (data not shown). This amino acid if part of the hydrophobic core structure of the third LRR and its substitution with the polar threonine residue destabilizes the protein domain fold (Nobile et al., 2009).

Family B has a single affected member, who was initially ascertained as a sporadic case. DHPLC analysis of *LGI1* exons revealed a sequence variation in exon 6 that was subsequently confirmed to be a mutation, *c.535T>C* (Fig. 1), which results in replacement of the cysteine at position 179 with an arginine (*C179R*). The patient inherited this mutation from his mother II-3; the mutation is also carried by his sister III-3 but was not found in 130 healthy controls. The C179 residue occurs at the second position in a cluster of four highly conserved cysteines ($CxCx_{20}Cx_{20}C$; x can be any amino acid) flanking the LRRs on the C-terminal side. The substitution of this amino acid with arginine inevitably causes a structural destabilization of the LRR domain, resulting from disruption of a disulfide bond that forms between residues C179 and C221 (see Nobile et al., 2009). The misfolded mutant protein is very likely retained within the cell and eliminated.

In family C, *LGI1* sequence analysis of the proband III-1 showed the nucleotide change *c.1075A>G* in exon 8 (Fig. 1), which entails a substitution of isoleucine at position 359 with valine (*I359V*). This substitution was not found, however, in the proband's cousin III-3, also affected with lateral temporal epilepsy, strongly suggesting that this is not a disease-causing mutation. Given that several species—including elephant, opossum, and chicken—have a constitutive V359 and that both isoleucine and valine are hydrophobic amino acids, this is likely to be a rare variant (it was not found in 130 healthy controls) with no effect on susceptibility to ADLTE/ADPEAF.

In family D, sequencing of *LGI1* exons in the patients IV-3 and IV-4 revealed a heterozygous *c.406C>T* transition in exon 4 (Fig. 1), giving rise to an arginine to tryptophan substitution at position 136 of the protein sequence (*R136W*). This mutation was previously reported to occur de novo in a sporadic case of telephone-induced partial epilepsy with typical lateral temporal lobe semiology (Michelucci et al., 2007). It eliminates an MspI restriction site. RFLP analysis of exon 4 yields two digested wild-type fragments of 356 and 109 bp or an undigested mutant allele of 465 bp (Fig. 1). The mutation was also found in patient III-3 and in the unaffected family members II-7, II-9, III-1, III-6, III-8, and III-9, but not in subjects III-4 and III-5 and in 130 unrelated healthy controls. The R136 residue is highly conserved. Replacement of this charged amino acid with the hydrophobic tryptophan hampers the function of the mutated protein (Nobile et al., 2009), ultimately resulting in the epilepsy phenotype.

Cell transfection assay

To ascertain the functional consequences of these non-synonymous variants, we transfected LGI1Flag cDNA constructs containing the mutations *c.365T>C, c.406C>T,* or the variant *c.1075A>G* into HEK293 cells, which do not express endogenous *LGI1*. A construct containing the wild-type sequence was also used as control. The proteins produced by transfected cells were then analyzed by immunoblot. Both cell lysates and concentrated (about 20×) conditioned media were analyzed using anti-Lgi1 and anti-Flag antibodies. The Lgi1 wild-type protein was detected in the medium as well as the lysate of transfected cells, whereas the I122T- and R136W-mutated proteins were detected only in the cell lysates (Fig. 2). Similar results were obtained with an additional LGI1 cDNA carrying the mutation *c.329C>A* (*A110D*) previously reported to cause ADLTE in an American family (Ottman et al., 2004). Instead, the protein containing the I359V variant showed the same secretion pattern as the wild-type protein (Fig. 2), further supporting the neutral nature of this variation. The effect of the *c.535T>C* (*C179R*) mutation on protein secretion was not tested because this mutation disrupts one of the disulfide bonds that stabilize the LRR domain structure, very likely resulting in intracellular elimination of the misfolded protein (see above, and Nobile et al., 2009).

Overall, the effects of the three variants tested on Lgi1 protein secretion were in agreement with functional predictions based on the PolyPhen and SIFT algorithms (Table 2). The negative effects of *I122T* and *R136W* on secretion were consistent with the ''probably damaging'' and ''not tolerated'' scores predicted by these two programs, respectively; whereas the absence of effect on protein secretion of

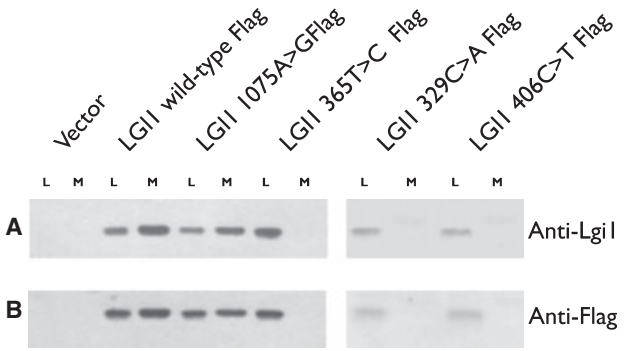

Figure 2.
Immunoblot analysis of transfected HEK293 cells. Cell lysates (L) and concentrated media (M) of HEK293 cells transfected with LGI1 wild-type Flag, LGI1 406C>T Flag, LGI1 365T>C Flag, and LGI1 1075A>G Flag expression constructs, or with empty expression vector (vector), were analyzed by western blot using either an anti-LGI1 (**A**) or an anti-Flag (**B**) antibody. An expression construct with the mutation c.329C>A (A110D; Ottman et al., 2004) was used as control.
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Table 2. Functional predictions and effects on secretion of newly identified variants

Variant	PolyPhen score[a]	SIFT score[b]	Grantham score[c]	Effect on secretion
I122T	2.29	0.00	89	Negative
C179R	3.83	0.00	180	NT
I359V	0.04	1.00	29	None
R136W	2.87	0.00	NP	Negative

NP, not predicted; NT, not tested.
[a]0.00–0.99, benign; 1.00–1.49, potentially damaging; 1.50–1.99, possibly damaging; ≥2.00, probably damaging.
[b]0.00–0.05, not tolerated; 0.06–0.20, potentially not tolerated; 0.21–1.00, tolerated.
[c]0–50, conservative; 51–100, moderately conservative; 101–150, moderately radical; ≥151, radical.

the *I359V* variant was in agreement with "benign" and "tolerated" predictions. On the other hand, predictions based on the Grantham matrix (Grantham, 1974) were less accurate, as the *I122T* variant was predicted to be "moderately conservative" and no prediction at all was made for *R136W* (Table 2).

Discussion

We have described four novel ADLTE/ADPEAF families with nonsynonymous sequence variants in the *LGI1* gene. Three of these variants (*R136W, C179R,* and *I122T*) are inherited pathogenic mutations, as supported by their (1) cosegregation with disease, (2) conservation of the affected amino acid, (3) predicted deleterious effect, and (4) absence in healthy controls. In addition, two mutations (*R136W*, and *I122T*) were shown to prevent protein secretion, further supporting their pathogenicity. On the other hand, *I359V* is likely to be a neutral variant because it does not cosegregate with disease, affects a poorly conserved amino acid, and is predicted to have no pathogenic effect by functional prediction programs. That this variant was not found in a cohort of 130 Italian healthy controls may be accounted for by the origin of all members of this family from a small town on the mountains in South Italy. It is, therefore, likely that *I359V* is a rare polymorphism restricted to that geographic area, as suggested by the fact that the minor allele was found also in subject II-4 in family C (Fig. 1). The neutral nature of the *I359V* variation is also supported by its inability to prevent protein secretion. All ADLTE-related *LGI1* mutations tested so far have been found to inhibit protein secretion (see Nobile et al., 2009), and this functional effect has been regarded as the main mechanism leading to haploinsufficiency (Senechal et al., 2005; Sirerol-Piquer et al., 2006). However, we recently found a familial *LGI1* mutation associated with a very peculiar clinical phenotype (including absence of auditory or aphasic auras in all affected family members) that was unable to prevent protein secretion (Striano et al., 2011). Whether these atypical clinical and cellular phenotypes are correlated is at present unknown. In any case, the absence of effect on protein secretion seems to be a relatively rare functional feature of pathogenic *LGI1* mutations—so far observed in only one of 15 mutations tested (Nobile et al., 2009)—and, therefore, a negative secretion test should be considered an good indicator of pathogenicity of *LGI1* mutations, especially useful if other predictive methods give ambiguous results.

A recent analysis of 24 *LGI1*-mutated families has yielded an overall estimate of 67% penetrance, and this figure does not vary according to mutation type or location within the gene (Rosanoff & Ottman, 2008). *LGI1* mutations with reduced penetrance (<50%) have been found in a small proportion (12.5%) of ADLTE/ADPEAF families (Rosanoff & Ottman, 2008). Two of the families described in this article segregate *LGI1* mutations with apparent low penetrance. In family D, only three of nine mutation carriers have epilepsy (33%). Even including the two unaffected obligate carriers II-2 and II-4, and considering the patient IV-2 as a likely carrier of the *R136W* mutation (Fig. 1), the family penetrance remains 33%. This penetrance estimate is reliable, since the age of all the unaffected mutation carriers is well above the age period of risk and, therefore, the probability for them to develop epilepsy later in their life is low. In addition, the occurrence of subtle manifestations of epilepsy in some members of this family is unlikely, as all members who had no seizures were specifically asked about auditory and other sensory (aphasic, visual, vertiginous, psychic, epigastric) phenomena but none was reported.

De novo *LGI1* mutations are found in about 2% of patients with lateral temporal epilepsy (LTE) with auditory symptoms (Bisulli et al., 2004; Michelucci et al., 2007).

The isolated patient of family B came to our attention as a potential sporadic LTE case. However, he received the *c.535T>C* mutation from his mother, as did one of his three sibs. Because there seems to be no other recognizable affected in the mother's family, this appears to be a pseudo-sporadic case of LTE caused by an inherited mutation with low penetrance. Therefore, families B and D confirm that certain *LGI1* mutations may occur with low penetrance in some ADLTE/ADPEAF families with apparent complex inheritance or even in some isolated cases. The number of ADLTE/ADPEAF families reported so far is still low; families with higher recurrence of auditory temporal epilepsy have more easily been identified and referred for genetic analysis. In these relatively large families, if mutated, highly penetrant *LGI1* mutations have frequently been found (Rosanoff & Ottman, 2008). As more ADLTE/ADPEAF families are identified and tested, the proportion of families with lower recurrence of the syndrome, which are more difficult to diagnose, will probably increase and detection of low penetrant *LGI1* mutations will likely become more common.

The *LGI1 R136W* mutation that segregates in family D is not novel. It was previously reported to occur de novo in a sporadic case of telephone-induced partial epilepsy with typical lateral temporal lobe semiology (Michelucci et al., 2007). Because it causes seizures without any triggering factors in this family, the *R136W* mutation seems to be responsible for the lateral temporal semiology rather than the pathophysiologic mechanisms underlying the reflex nature of telephone-induced seizures.

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Disclosure

None of the authors has any conflict of interest to disclose. 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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