A targeted resequencing gene panel for focal epilepsy

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ABSTRACT

Objectives: We report development of a targeted resequencing gene panel for focal epilepsy, the most prevalent phenotypic group of the epilepsies.

Methods: The targeted resequencing gene panel was designed using molecular inversion probe (MIP) capture technology and sequenced using massively parallel Illumina sequencing.

Results: We demonstrated proof of principle that mutations can be detected in 4 previously genotyped focal epilepsy cases. We searched for both germline and somatic mutations in 251 patients with unsolved sporadic or familial focal epilepsy and identified 11 novel or very rare missense variants in 5 different genes: *CHRNA4*, *GRIN2B*, *KCNT1*, *PCDH19*, and *SCN1A*. Of these, 2 were predicted to be pathogenic or likely pathogenic, explaining ~0.8% of the cohort, and 8 were of uncertain significance based on available data.

Conclusions: We have developed and validated a targeted resequencing panel for focal epilepsies, the most important clinical class of epilepsies, accounting for about 60% of all cases. Our application of MIP technology is an innovative approach that will be advantageous in the clinical setting because it is highly sensitive, efficient, and cost-effective for screening large patient cohorts. Our findings indicate that mutations in known genes likely explain only a small proportion of focal epilepsy cases. This is not surprising given the established clinical and genetic heterogeneity of these disorders and underscores the importance of further gene discovery studies in this complex syndrome. *Neurology*® 2016;86:1605–1612

GLOSSARY

ExAC = Exome Aggregation Consortium; **MIP** = molecular inversion probe; **NFLE** = nocturnal frontal lobe epilepsy; **smMIP** = single molecule molecular inversion probe; **TLE** = temporal lobe epilepsy.

Investigation of genetic epilepsies has proven highly successful in recent years in a subset of cases. A variety of genes that cause human epilepsies have been discovered, revealing novel and unexpected biological pathways and providing important diagnostic and counseling information for patients and their families.¹ Focal epilepsies account for about 60% of all epilepsies.^{2,3} Despite being the most common form of epilepsy, focal epilepsies had been definitively associated with only 11 genes at the time of platform design, mostly as a result of genetic analysis of large multiplex families. De novo mutations (likely occurring in parental gametes) of a number of genes have been discovered,^{4–7} indicating genetic causation in patients with focal epilepsy even without a family history. In this study we sought to determine the contribution of known genes to the causation of a broad spectrum of focal epilepsies.

To unravel this contribution, we studied a large resource of patients with focal epilepsy (n = 251). The phenotypic and genetic heterogeneity of focal epilepsies warranted a comprehensive targeted resequencing approach. We applied single molecule molecular inversion probes (smMIPs⁸) to focal epilepsies, arguably the most clinically important group of epilepsies, to develop a focal epilepsy targeted resequencing gene panel.

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METHODS **Standard protocol approvals, registrations, and patient consents.** The Human Research Ethics Committee of Austin Health, Melbourne, Australia, and the Institutional Review Board of University of Washington Medical Center approved this study. Informed consent was obtained from living patients or their relatives.

Patient phenotyping. Patients with focal epilepsy without a known acquired cause were recruited from the first-seizure and epilepsy clinics at Austin Health, from the private practices of the investigators, and by referral for genetics research over a period of 25 years, regardless of reported family history of epilepsy. Clinical data and a detailed family history were obtained using a validated seizure questionnaire and personal evaluation and review of medical records, including EEG and neuroimaging investigations.⁹ MRI (generally 1.5T) was performed in 243 of 251 patients and showed no acquired lesion (tumor, trauma, stroke, etc.). Hippocampal sclerosis was allowed because although it may follow prolonged febrile convulsion or cerebral infection, a heritable component has been reported, including mutations in the *SCN1A*¹⁰ and *SCN1B*¹¹ genes in a small number of cases. We included 14 patients with hippocampal sclerosis; none had prior cerebral infection and only 2 had prior febrile seizures. Patients that did not receive MRI had normal CT scans and no history of an acquired insult.

In some cases, further evaluation and follow-up was performed as necessary.¹² Cases were considered “familial” if they reported a first-degree relative affected with epilepsy. All available family members were included in the segregation analysis for variants of interest. In some cases, parents or other family members were unavailable for segregation analysis because of death, separation, or loss of contact.

Sample preparation. For most samples, whole venous blood was obtained and genomic DNA extracted using a Qiagen QIAamp DNA Maxi Kit (Valencia, CA) according to the manufacturer’s instructions and as described previously.¹³ In some cases, only saliva samples were available, and DNA was extracted from these specimens using a prepIT•L2P kit (DNA Genotek Inc, Ontario, Canada) according to the manufacturer’s instructions.

Gene selection. We selected 11 genes reported to cause focal epilepsy syndromes for capture and sequence analysis: *CHRNA2*, *CHRNA4*, *CHRNA2*, *DEPDC5*, *GRIN2A*, *GRIN2B*, *KCNT1*, *LGII*, *PCDH19*, *SCN1A*, and *TBCID24*.^{4,5,14–20}

Targeted resequencing and analysis. We designed smMIPs to capture all exon and intron-exon boundaries (5-bp flanking sequences) of target genes (RefSeq, hg19 build). Detailed methodology is described elsewhere.⁸ Briefly, pooled smMIPs that included unique 5-nucleotide molecular tags for detection of low-frequency or subclonal variation were used to capture target exons from 100 ng of DNA from each proband. PCR

was performed using universal primers, with the introduction of an 8-nucleotide barcode on the tagged reverse primer to uniquely identify each proband. Pooled libraries were subjected to massively parallel sequencing using a 100 paired-end protocol on the HiSeq platform. Libraries of the 11 captured genes from 312 samples were prepared and sequenced in 1 batch. Of the 312 samples prepared, 81.2% passed our quality control benchmark, namely that each sample have a minimum of 70% of bases covered by at least 5 unique capture events (table e-1 on the *Neurology*[®] Web site at Neurology.org).

Raw read data processing and mapping with Burrows-Wheeler Aligner and single nucleotide variant and indel calling and filtering using the Genome Analysis Toolkit were performed as previously described.⁸ Variants meeting the following criteria were excluded from further analysis: clustered variants (window size of 10) and those variants with an allele balance >0.75, quality <30, quality by depth <5, or unique capture events <5. Variants were annotated with SeattleSeq (v137), and the Exome Sequencing Project (ESP6500), 1000 Genomes Project, and Exome Aggregation Consortium (ExAC) (v0.3) data sets were used to assess variant frequency in the control population. Only nonsynonymous, splice-site, stop-gain, and frameshift variants with a minor allele frequency <0.1 were assessed further. For dominant (or de novo) models, we considered only variants not present in these control data sets.

Rare variant analysis. Variants were validated using a MIP-pick strategy or PCR and Sanger sequencing. For the former, we selected and pooled only the MIPs that captured the genomic sequence harboring the rare variant of interest and performed targeted resequencing as described above. This approach allowed us to sequence variants at very high depth. For the latter, we performed PCR and Sanger sequencing to analyze likely heterozygous variants. Gene variants were amplified using gene-specific primers (oligonucleotides available on request) designed to the reference human gene transcripts (NCBI Gene; <http://www.ncbi.nlm.nih.gov/gene>). Amplification reactions were cycled using a standard protocol on a Veriti Thermal Cycler (Applied Biosystems, Carlsbad, CA) at 60°C annealing temperature for 1 minute. Bidirectional sequencing of all exons and flanking regions was completed with a BigDye v3.1 Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer’s instructions. Sequencing products were resolved using a 3730xl DNA Analyzer (Applied Biosystems). All sequencing chromatograms were compared to published complementary DNA sequence; nucleotide changes were detected using Codon Code Aligner (CodonCode Corporation, Dedham, MA).

Criteria for pathogenicity of rare or novel variants. We classified rare or novel variants as pathogenic, likely pathogenic, of uncertain significance, or benign. Pathogenic or likely pathogenic variants had to (1) be very rare (e.g., present in ≤5 alleles in ~63,000 exomes of ExAC database and no homozygotes reported), (2) arise de novo or segregate with the disorder, (3) be predicted to damage an important protein domain, and (4) be associated with an established epilepsy phenotype for the given gene. Variants failing to meet these criteria were considered of uncertain significance or benign.

RESULTS **Validation of focal epilepsy panel.** In total, 251 patients and 4 positive controls (table 1; table e-1) were studied, meaning 255 individuals underwent genetic analysis. The majority of patients (n = 151) had temporal lobe epilepsy (TLE), the most common adult-onset focal epilepsy syndrome.²¹ The remainder

Table 1 Positive control probands

Proband	Syndrome	Gene	Mutation	Reference
T486	NFLE	KCNT1	c.1193G>A (p.Arg398Gln)	5
T18469	NFLE	KCNT1	c.2688G>A (p.Met896Ile)	5
T832	NFLE	KCNT1	c.2782C>T (p.Arg928Cys)	5
4046	FFEVF	DEPDC5	c.21C>G (p.Y7X)	4

Abbreviations: FFEVF = familial focal epilepsy with variable foci; NFLE = nocturnal frontal lobe epilepsy.

Table 2 Coverage statistics for focal epilepsy panel

Gene	Average unique captures per base across all samples
<i>CHRNA2</i>	80.7
<i>CHRNA4</i>	38.8
<i>CHRN2</i>	62.0
<i>DEPDC5</i>	108.3
<i>GRIN2A</i>	92.0
<i>GRIN2B</i>	96.3
<i>KCNT1</i>	60.1
<i>LGI1</i>	69.0
<i>PCDH19</i>	45.5
<i>SCN1A</i>	97.9
<i>TBC1D24</i>	62.3

had less common forms of epilepsy including frontal lobe epilepsy and mesial temporal lobe epilepsy with hippocampal sclerosis (table e-1). The cohort comprised largely sporadic cases ($n = 200$), although 51 cases were “familial” (report of a first-degree relative affected with epilepsy). Of these, 4 relatives had epilepsy that was likely of nongenetic etiology, for example posttraumatic epilepsy or epilepsy associated with a brain tumor.

Overall, 88% of the target (11 genes) was uniquely captured at least 5 times and sequenced at $>50\times$ coverage, which is required for accurate variant calling, as described previously²² (table 2; tables e-1, e-2, and e-3; figure 1). All 4 pathogenic variants that had previously been detected from samples in research studies were validated (table 1); these cases were not included in the discovery cohort.

Novel or very rare variant discovery. We searched for heterozygous and low-frequency variation in the 11 focal epilepsy genes among our 251 patients with focal epilepsy. Novel or very rare variants were present in 5 of 11 genes assayed (table 3). Our variant analysis revealed 27 high-confidence heterozygous missense variants, 26 of which were validated by MIP-pick or Sanger sequencing. Of the 26 heterozygous missense variants, 11 were novel or very rare according to the ExAC database and were investigated further. Of these, 2 were classified as pathogenic or likely pathogenic, 8 as of uncertain significance, and 1 as benign (table 3).

***KCNT1*.** Three novel variants were found in *KCNT1* (table 3), of which only 1, p.Arg950Gln, was in a patient (9092) with nocturnal frontal lobe epilepsy (NFLE), a phenotype definitively associated with this gene.⁵ Analysis of his parents revealed that this is a de novo mutation located in the cytoplasmic domain that is predicted to be damaging to the channel protein (figure 2). Functional assays that will be reported elsewhere have confirmed that this mutation activates *KCNT1* channels similarly to previously reported NFLE-associated mutations.²³ The other 2 variants (in T2957 and T2958; table 3) are of uncertain significance at this time based on the TLE phenotype and unavailability of parental samples for segregation.

***GRIN2B*.** The phenotypes associated with *GRIN2B* mutations include West syndrome, Lennox-Gastaut syndrome, a single case of mild intellectual disability and focal epilepsy from age 9 years, and a few cases of varying degrees of intellectual disability with no report of seizures.^{24–27} The p.Glu47Gly variant present in T22312 (figure 2, table 3) affects a highly conserved glutamate residue that may be involved in localizing water molecules around the Zn1 zinc-binding site of

Figure 1 Molecular inversion probe capture events

Percent of targeted genes covered by at least 5 unique capture events.

Table 3 Very rare and novel variants detected in focal epilepsy cases

Proband (age, y, sex)	Gene	GRCh37/hg19	Protein change; domain	ExAC	PolyPhen-2	SIFT	CADD	Epilepsy syndrome	Seizure types (onset age, y)	Diurnal/nocturnal seizures?	EEG	Epileptogenic lesion	Other features	First-degree relative affected with epilepsy?	Segregation	Pathogenicity prediction
9092 (29, M)	KCNT1	chr9:138675877G>A	p.Arg950Gln; cytoplasmic	0 times	Possibly damaging	Deleterious	33.0	NFLE	Aura (3); FDS (13)	Mostly nocturnal	Interictal independent L temporal, R temporal, R frontal, and L frontal discharges; ictal recording shows a bifrontal onset	N	Mild ID, psychosis, depression, inertia, psychomotor retardation	N	De novo	Pathogenic
T20252 (21, F)	SCN1A	chr2:166894513C>A	p.Val907Phe; transmembrane	0 times	Probably damaging	Deleterious	27.3	Lateral TLE	Aura (15); FDS (15); BC (17)	Both	Bitemporal slow waves	N	Depression	N	Mother wild-type; father unavailable	Likely pathogenic
T3566 (35, F)	CHRNA4	chr20:61981883C>T	p.Glu294Lys; transmembrane	1 time	Probably damaging	Deleterious	32.0	TLE/OLE	BC (12); FDS (16); aura (>16)	Both	PPR (GPSW), bitemporal slowing R > L	N	Syncope, depression	N	Parents unavailable	Uncertain significance
T2033 (41, F)	GRIN2B	chr12:13828696C>T	p.Glu370Lys; extracellular	0 times	Benign	Deleterious	17.0	TLE	BC (11); aura (>11)	Both	Bilateral temporal sharp waves R > L	N	Nil	N	Mother wild-type; father unavailable	Uncertain significance
T2958 (29, F)	KCNT1	chr9:138678185G>A	p.Arg1107His; cytoplasmic	0 times	Benign	Tolerated	10.5	TLE	Prolonged FS (9 mo); aura (7); FDS (16)	Both	Right temporal epileptiform (ictal recording)	Right HS	Anxiety, depression	N	Parents unavailable	Uncertain significance
T2957 (55, M)	KCNT1	chr9:138642851G>A	p.Arg133His; extracellular	0 times	Probably damaging	Deleterious	18.4	TLE	FDS (45); BC (45)	Both	Left temporal epileptiform (ictal recording)	N	Nil	N	Parents deceased	Uncertain significance
T17190 (52, M)	PCDH19	chrX:99661680T>G	p.Glu639Ala; cadherin	1 time (F)	Possibly damaging	Deleterious	15.8	TLE	Aura (25); FDS (45); BC (45)	Diurnal	Normal	N	Psychiatric disorder	Y	Parents deceased	Uncertain significance
T3047 (34, M)	PCDH19	chrX:99551564G>A	p.Arg1053Gln; cytoplasmic	0 times	Possibly damaging	Deleterious	19.0	Lateral TLE	BC (19); FDS (32); aura (33)	Diurnal	R temporal slowing	N	Nil	N	Mother unavailable; father deceased	Uncertain significance
T20319 (15, F)	PCDH19	chrX:99551748C>T	p.Ala992Thr; cytoplasmic	1 time (M)	Benign	Tolerated	6.5	FLE	FDS (11); BC (14)	Both	Interictal central and L parasagittal epileptiform; ictal low voltage fast activity in the centroparietal region	N	Global DD, depression, dyspraxia	N	Parents unavailable	Uncertain significance

Continued

Table 3 Continued

Proband (age, y, sex)	Gene	GRCh37/hg19	Protein change; domain	ExAC	PolyPhen-2	SIFT	CADD	Epilepsy (onset age, y)	Seizure types	Diurnal/nocturnal seizures?	EEG	Epileptogenic lesion	Other features	First-degree relative affected with epilepsy?	Segregation	Pathogenicity prediction
T22312 (37, F)	GRIN2B	chr12:14019003T>C	p.Glu47Gly; extracellular	1 time	Benign	Deleterious	17.9	TLE	Aura (26); BC (36)	Both	Postictal slowing, bitemporal maximum	N	Nil	N	Inherited from unaffected father	Uncertain significance
	SCN1A	chr2:166894306T>G	p.Met976Leu; transmembrane times	3	Benign	Tolerated	16.0								Inherited from unaffected father	Benign

Abbreviations: BC = bilateral convulsive seizure; DD = developmental delay; FDS = focal dyscognitive seizure; FLE = frontal lobe epilepsy; FS = febrile seizure; GPSW = generalized poly-spike and wave; HS = hippocampal sclerosis; ID = intellectual disability; NFLE = nocturnal frontal lobe epilepsy; PPR = photo paroxysmal response; TLE = temporal lobe epilepsy; TLE/OLE = temporo-occipital lobe epilepsy.

the GRIN2B amino terminal domain.²⁸ It has also been shown that mutating Glu47 to alanine reduced zinc sensitivity by 4-fold.²⁹ This mutation is predicted to lead to a hyperactive receptor, as NMDA receptor activity is inhibited by zinc, but the significance of the mutation in patient T22312 is currently unknown. The p.Glu370Lys variant in patient T2033 is also located in the extracellular amino terminal domain of GRIN2B. This is a novel variant in a less studied sub-domain, making it a variant of uncertain significance at this time.

SCN1A. Both *SCN1A* variants were in patients with TLE. Although *SCN1A* has been described in patients with mesial TLE, 1 patient, T20252, had lateral TLE, which was associated with *SCN1A* only recently.^{10,30,31} No history of febrile seizures was reported in the extended family of patient T20252. Both variants in this study are located within transmembrane domains. The novel p.Val907Phe variant in T20252 is predicted by in silico software to be damaging to the SCN1A protein. We have limited inheritance information for this proband because the mother is not a carrier and a paternal DNA sample was not available (figure 2, table 3). We have thus designated this variant as likely pathogenic. The other SCN1A variant, p. Met976Leu, is predicted to be tolerated, present in the ExAC database in 3 individuals, and therefore likely benign. This same individual, T22312, is also a heterozygous carrier of a rare GRIN2B variant (p.Glu47Gly) of unknown significance (figure 2, table 3).

PCDH19. There were 3 *PCDH19* variants of unknown significance. The first, p.Ala992Val, is present in female patient T20319 but is located in the cytoplasmic domain and is not predicted to be damaging. This variant has never been reported in a female but was observed once in the ExAC data set in a single male. Another variant, p.Arg1053Gln, is a novel variant present in male patient T3047. This variant is in the cytoplasmic domain and may be damaging (CADD 19.03, PolyPhen-2 possibly damaging, SIFT deleterious). The third variant, p.Glu639Ala, is located in the cadherin domain, where other mutations linked to *PCDH19*-related epilepsy have been found, but the patient, T17190, is male. Of note, his brother who also has been diagnosed with TLE also carries the variant (figure 2, table 3). Although *PCDH19*-related epilepsy mainly affects females, there is some evidence that males may also be affected if mosaicism is involved.³²

CHRNA4. The *CHRNA4* p.Glu294Lys variant in patient T3566 with TLE is located in a transmembrane domain and is predicted to be damaging. This change from an acidic residue to a basic residue, especially at such a critical domain, is likely to impair channel function, but mutations in this gene have

Figure 2 Segregation analysis

Pedigrees of extended families with family members available for sequencing showing segregation of variants.

been linked only to NFLE.²⁰ The pGlu294Lys is a variant of uncertain significance at this time. *CHRNA2* and *CHRNA4* were also included on the panel; despite having adequate coverage, there were no variants of interest to report in these genes from this study.

DISCUSSION Our large collection of focal epilepsy cases afforded an ideal opportunity to rigorously investigate the clinical significance of the known focal epilepsy genes. As a first step toward implementing an efficient and cost-effective clinical test using MIP capture technology, we developed a focal epilepsy

panel including the 11 genes causally linked to this common disorder. We demonstrated the feasibility of this panel on 4 positive controls and 251 patients with sporadic and familial focal epilepsy. We identified pathogenic or likely pathogenic variants in 2 of 251 patients, explaining ~0.8% of the cohort. One reason for the low hit rate is the likelihood of ascertainment bias because patients were selected retrospectively and a subset had had previous negative screening for some focal epilepsy genes (e.g., *KCNT1*, *DEPDC5*).

It is also noteworthy that 8 of the 11 very rare or novel variants we discovered (table 3) had uncertain significance based on available clinical and molecular genetic data. This highlights the challenges associated with determining the significance of gene variants discovered in multigene screens, particularly for syndromes such as focal epilepsy in which most of the heritability is unexplained. By chance, all 11 variants were missense changes, and definitively determining the pathogenicity of this variant class is often problematic. Strategies for classifying variants have been reported, including a recent set of guidelines³³ that were the basis for the classification used herein. These challenges will continue to bedevil the field in the near future, and phenotype–genotype correlations will be critical to determining relevance, as will large curated databases of clinical and molecular information.

Despite using the multiplex smMIPS platform that detects heterozygote mutations and low-level mosaic variants, we did not confirm any somatic mosaic changes. It was difficult to predict the number of somatic mutations that might contribute because the contribution of somatic mutations to the genetic architecture of this common group of epilepsies is not known. Nonetheless, given the clinical severity of many focal epilepsies, their focal origin, and their later onset in comparison to the genetic generalized epilepsies, it is not unreasonable to assume that a non-negligible number of pathogenic mutations are somatic, arise later in development, and have expression restricted to brain tissue. This latter issue may in part explain our lower-than-expected hit rate. Copy number variants may also contribute to the genetic etiology but were not systematically evaluated in this study. In parallel studies that take us directly from molecular diagnosis using our focal epilepsy panel to the bedside, patient 9092 has been enrolled in a clinical trial to test the efficacy of a US Food and Drug Administration–approved reversible *KCNT1* channel-blocking drug. This exemplar illustrates the power of molecular diagnosis to directly inform clinical management in focal epilepsy.

AUTHOR CONTRIBUTIONS

S.F.B. and H.C.M. initiated the project. M.S.H., C.T.M., S.F.B., and H.C.M. directed the project. M.S.H., C.T.M., and G.L.C. performed

MIPs capture and sequencing. C.T.M. and G.L.C. performed sequence analysis. C.T.M. performed variant validation by MIP-picking. M.S.H. and J.A.D. performed variant validation by Sanger sequencing. B.M.R., S.A.M., M.R.N., I.E.S., and S.F.B. conducted clinical phenotyping. E.V.G., U.N., C.J.M., C.A.R., and S.P. performed electrophysiology on *KCNT1* variants. M.S.H., C.T.M., S.F.B., I.E.S., and H.C.M. wrote the paper. All authors discussed the results and commented on the manuscript.

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