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Diagnostic Targeted Resequencing in 349 Patients with Drug-Resistant Pediatric Epilepsies Identifies Causative Mutations in 30 Different Genes

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ABSTRACT: Targeted resequencing gene panels are used in the diagnostic setting to identify gene defects in epilepsy. We performed targeted resequencing using a 30-genes panel and a 95-genes panel in 349 patients with drug-resistant epilepsies beginning in the first years of life. We identified 71 pathogenic variants, 42 of which novel, in 30 genes, corresponding to 20.3% of the probands. In 66% of mutation positive patients, epilepsy onset occurred before the age of 6 months. The 95-genes panel allowed a genetic diagnosis in 22 (6.3%) patients that would have otherwise been missed using the 30-gene panel. About 50% of mutations were identified in genes coding for sodium and potassium channel components. *SCN2A* was the most frequently mutated gene followed by *SCN1A*, *KCNQ2*, *STXBP1*, *SCN8A*, *CDKL5*, and *MECP2*. Twenty-nine mutations were identified in 23 additional genes, most of them recently associated with epilepsy. Our data show that panels targeting about 100 genes represent the best cost-effective diagnostic option in pediatric drug-resistant epilepsies. They enable molecular diagnosis of atypical phenotypes, allowing to broaden phenotype–genotype correlations. Molecular diagnosis might influence patients' management and translate into better and specific treatment recommendations in some conditions.

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KEY WORDS: epilepsy; next-generation sequencing; gene panel; mutation

Introduction

Many epilepsies and epilepsy syndromes have genetic causes [Gourfinkel-An et al., 2004; Guerrini et al., 2006; Helbig et al., 2008]. Recent whole exome and genome sequencing studies focussing on monogenic severe epilepsies and epileptic encephalopathies (EEs) have indeed identified mutations in many genes [Epi4k Consortium, 2013; Myers and Mefford, 2015; Helbig et al., 2016]. The hypothesis of one gene-one disease has proven to be incorrect for most syndromes, thus clinicians standstill with phenotypes that might overlap but are associated with mutations in different genes or might confront with a spectrum of phenotypes being caused by mutations in the same gene [Carvill et al., 2013; Epi4k Consortium, 2013]. Loose genotype–phenotype correlations place the clinician in the difficult position of not knowing the most suitable candidate gene that might underlie the epilepsy afflicting the young patient. Therefore, targeted resequencing of selected genes (gene panels) appears to be the best cost-effective diagnostic option. Recent studies have indeed shown that gene panels have the power of reaching a diagnosis in about 20% of probands with severe epilepsies and developmental delay [Trump et al., 2016] and such proportion might increase up to nearly 50% when the number of genes included in the panel is very high and patients analyzed have a spectrum of hypothetically genetic epilepsies [Lemke et al., 2012].

This study was conceived to elaborate on clinical and genetic data of 349 patients with pediatric drug-resistant epilepsies analyzed using targeted resequencing (next-generation sequencing; NGS) with an initial panel of 30 genes and a second larger panel of 95 genes, or both for a subset of patients. The panels include major epilepsy genes and also genes that are not frequently analysed by standard methods. We identified 71 pathogenic variants corresponding to 20.3% of the probands. Most mutations occurred in probands with epilepsy onset before the age of 6 months and laid in known epilepsy genes. *SCN2A* was the most frequently mutated gene in 2.6% of the cohort followed by *SCN1A*, *KCNQ2*, *STXBP1*, *SCN8A*, *CDKL5*, and *MECP2*. The larger panel identified additional mutations in rare genes and patients here described contribute to delineate the associated, yet uncertain phenotypes. Thus, this study contributes to confirm the utility of NGS gene panels in a clinical diagnostic setting and the usefulness of using panels comprehensive of both well-known and rarely associated epilepsy genes, the later enabling molecular diagnosis of atypical phenotypes.

Additional Supporting Information may be found in the online version of this article.

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Materials and Methods

We assembled a cohort of 349 consecutive patients, with no obvious developmental or acquired brain abnormalities on 1.5T to 3T MRI or dysmorphic features, analyzed with NGS panels in our Neurogenetics Laboratory from 2013 to 2015. Patients were referred from the Pediatric Neurology Unit of the Anna Meyer Children's Hospital, as well as from other national and European epilepsy/neurology centers, and exhibited a wide spectrum of pediatric drug-resistant epilepsies. We defined epilepsy as "drug-resistant" when adequate trials of two tolerated, appropriately chosen, and used antiepileptic drugs schedules (whether as monotherapies or in combination) failed to achieve sustained seizure freedom [Kwan et al., 2010; Tellez-Zenteno et al., 2014]. We classified seizure types and epilepsy/syndromes according to the ILAE guidelines [Commission on Classification and Terminology, 1989; Berg et al., 2010]. Based upon age of seizures onset, we defined patients as having: (a) neonatal onset epilepsy when seizures occurred within the 1st month; (b) infantile epilepsy when presenting from the 1st to the 12th month; (c) childhood epilepsy when presenting after the 1st year of life [Berg et al., 2010]. Within the infantile epilepsies, patients with onset before 6 months of age are commonly referred to as having an early infantile epilepsy.

NGS was performed in 207 patients with severe epilepsies (Cohort A), predominantly either neonatal or infantile EEs, using a panel of 30 known epilepsy genes. NGS was performed in a cohort of 142 probands (Cohort B) with a broader spectrum of drug-resistant epilepsies using a larger panel targeting 95 genes associated with epilepsy. The genes targeted by these panels are listed in Supp. Table S1 and Supp. Table 2S. 34 out of the 207 patients of the Cohort A, who resulted mutation negative to the 30-genes panel, were also analyzed with the 95-genes panel.

We obtained approval for this study from the Institutional Review Board of the Meyer Children's Hospital. We obtained clinical information and blood/DNA samples after informed consent.

DNA was extracted from peripheral blood leukocytes using a QiaSymphony SP robot (Qiagen, Hilden, Germany) according to the manufacturer's protocol. High-quality DNA was quantified using a Quantifluor Fluorometer (Promega, Madison, WI).

A subset of patients, prior to NGS, underwent Sanger sequencing for one or more genes (Supp. Table S3). Array-CGH was performed in 109 patients (109/349: 31%).

30-Genes Panel Target Resequencing Analysis

The panel was designed using a custom target in solution enrichment NimbleGen SeqCap EZ Choice Library (Roche Inc., Madison, WI) to target the complete genomic sequence of selected genes, as well as the flanking regions at the 5′ and 3′ ends of each gene, accounting for a total of 109,528 bp. gDNA (500 ng) was nebulized and the libraries prepared using a GS FLX Titanium Rapid Library Preparation Kit (Roche Inc.). The libraries were multiplexed using different MID identifiers in order to analyze up to 12 samples in a single sequencing run, and the pool was hybridized to SeqCap EZ Choice Library designed to capture the genes included in the panel. Sequencing was performed according to the Roche FLX Titanium protocols and kits. Briefly, captured sample libraries were subjected to emulsion-based clonal amplification. DNA-carrying beads were enriched and used as template for sequencing by synthesis using the Titanium chemistry (XLR70 GS FLX Titanium sequencing kit; Roche Inc.). GS FLX sequence reads were aligned to the NCBI37/hg19 reference genome using the GS Reference Mapper v2.9 toolkit. Variants were called using the same toolkit. Exploiting the long reads generated by the GS FLX sequencer, we used the GS Reference Mapper to unravel potential structural rearrangements in the 30-genes panel. As we identified only two rearrangements involving the *STXBP1 and MECP2* genes, we subsequently confirmed them by Multiplex Ligation-dependent Probe Amplification (MLPA) as MLPA kits were available for both genes (SALSA MLPA probemix P138 *SLC2A1-STXBP1* and P015-F1 *MECP2*). For MLPA data analysis, we used the Coffalyser.Net tool (MRC-Holland).

95-Genes Panel Target Resequencing Analysis

The Haloplex panel was designed using the Agilent SureDesign tool (https://earray.chem.agilent.com/suredesign/index.htm) to capture the 95 epilepsy genes. gDNA was purified and resuspended in water using the DNA Clean & Concentrator-5 columns (Zymo Research Corporation, Irvine, CA) and the libraries prepared using the Haloplex target enrichment system (Agilent Technologies, Santa Clara, CA) according to the manufacturer instructions. Probes were generated to cover all coding exons and their flanking intronic sequences (10 base pairs padding). In brief, 225 ng of genomic DNA was used for restriction reactions, and hybridization with the Haloplex probe was performed for 3 hr at 54°C. Twelve libraries containing unique identifiers were quality controlled using a 2100 Bioanalyzer (Agilent Technologies), pooled in equimolar concentration and sequenced on a MiSeq sequencer using a MiSeq Reagent Kit v3 and a 150 bp paired-end chemistry (Illumina, San Diego, CA). Sequence reads were aligned to the NCBI37/hg19 reference genome using a pipeline based on BWA [Li and Durbin, 2009] and Picard (https://broadinstitute.github.io/picard/). Variants were called using the GATK toolkit [McKenna et al., 2010].

Variants Annotation and Filtering

For both panels, variants were annotated with gene name and classified according to their position and effect (frameshift, truncating, splicing, coding non synonymous, coding synonymous, intronic) using the ANNOVAR tool [Yang and Wang, 2015]. Variants localized in intronic regions outside the 10 bp exon flanking boundaries and in the 5'- and 3'-UTR regions were excluded. Variants reported in the Exome Aggregation Consortium (ExAC) database (http://exac.broadinstitute.org/) and/or in the 1000 Genomes Project (http://www.1000genomes.org) and/or in the NHLBI Exome Sequencing Project (ESP, ESP6500 database, http://evs.gs.washington.edu/EVS), with a Minor Allele Frequency > 0.01 (1%) were dropped out. *In silico* prediction of mutations' pathogenicity were obtained using ANNOVAR and the dbNSFP database (v3.0a), which provides functional prediction scores on more than 20 different algorithms (https://sites.google.com/site/jpopgen/dbNSFP). To assess the effects of missense substitutions, we used both the dbNSFP ensemble rank scores MetaSVM and MetaLR (Liu et al., 2016).

The cDNA numbering uses +1 as the A of the ATG translation initiation codon in the reference sequence, with the initiation codon as codon 1.

Variants Confirmation

Putative causative variants were analyzed by Sanger sequencing to confirm the NGS results and investigated in the parents of probands to check their inheritance status. The exons covering the coding

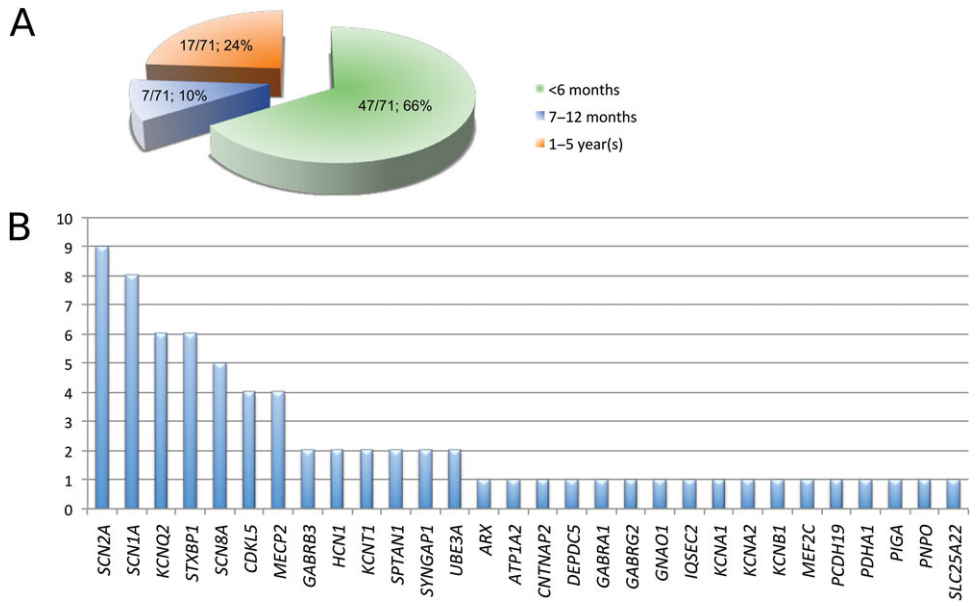

Figure 1. **A**: Graphic representation of the age at seizure onset of the 71 patients carrying pathogenic mutations: 47 patients (66%) had their first seizure prior to 6 months of age; seven patients (10%) had their first seizure between 7 and 12 months of age; 17 patients (24%) had their first seizure between 1 and 5 years of age. **B**: Graphic representation of the number of patients (*y*-axis) carrying mutations in 30 epilepsy genes (*x*-axis).

regions flanking the variants were amplified by PCR. PCR products were cycle sequenced on both strands using the BigDye Terminator v 3.1 chemistry (Applied Biosystems, Foster City, CA) and run on a 3130XL genetic analyzer (Applied Biosystems).

Criteria for Pathogenicity of Rare or Novel Variants

We classified rare or novel variants as being "pathogenic," "likely pathogenic," "variant of uncertain significance (VOUS)," "likely benign" or "benign," according to the international guidelines of the ACMG Laboratory Practice Committee Working Group [Richards et al., 2015].

We confirmed relatedness within families using the Powerplex Fusion Kit (Promega) when de novo mutations occurred.

Results

The study included 349 patients analyzed with NGS panels. We obtained detailed clinical information on age at seizures onset and epilepsy/syndrome type in 300 out of the 349 probands (86%) whose median age at seizure onset was 6 months (from 1st day of life to 12 years; mean 18.5 months). Probands were classified as having severe pediatric epilepsies with variable phenotypes, including well established syndromes such as West (57 probands), myoclonic-astatic epilepsy (MAE) (10 probands), Landau-Kleffner (eight probands), Ohtahara (seven probands), Dravet (DS) (six probands), electrical status epilepticus in sleep (four probands), Lennox-Gastaut (three probands), malignant migrating partial seizures of infancy (MMPSI) (two probands), Angelman (two probands), and Rett (two probands). The remaining patients presented less-defined phenotypes broadly classified, according to seizure type and onset, as: neonatal EE (37 probands), early infantile EE (44 probands), late infantile EE (20 probands), childhood EE (13 probands), neonatal or infantile or childhood drug-resistant focal or multifocal epilepsies (two, 15, and 31 probands), drug-resistant myoclonic absences (six probands), drug-resistant absence epilepsy (five probands), generalized epilepsies with febrile, and afebrile seizures (26 probands). There were 49 patients with a diagnosis of refractory pediatric epilepsy referred for "epilepsy genes panel screening" for whom information on age at seizures onset and epilepsy/syndrome type could not be retrieved. Supplementary table (Supp. Table S4) summarizes ages at seizure onset (median, mean, and range) and epilepsy type/syndrome of the 300 patients (Group A = 30-gene panel; Group B = 95-gene panel or both—in brackets). The only differences between the two groups were a wider age range at epilepsy onset and a slight predominance of neonatal and early infantile EE in Group A and of MAE and genetic generalized epilepsy in Group B.

The 349 patients were analyzed with NGS panels of 30 genes, 95 genes, or both. Eighty-six patients (24.6%) harbored novel or rare variants, 71 (20.3%) of these variants were classified as "pathogenic" or "likely pathogenic" mutations, whereas 15 (4.3%) were classified as VOUS. Forty-two mutations were novel.

Using the 30-genes panel in Cohort A (207 patients), we obtained a mean coverage of 95% bases covered at ≥10× and we found 40 patients (40/207: 19.3%) to carry novel or rare variants classified as "pathogenic" or "likely pathogenic". Using the 95-genes panel for the analysis of Cohort B (142 patients), we obtained a mean coverage of 98% bases covered at ≥30× and we found 26 patients (26/142: 18.3%) to carry novel or rare variants classified as "pathogenic" or "likely pathogenic". Thirty-four patients, who were mutation-negative after the 30-genes panel screening, were subsequently analyzed with the 95-genes panel. This analysis identified five *de novo* mutations (5/34: 14.7%), four of which involving genes not included in the 30-genes panel. The c.3690dupT [p. (Ser1231∗)] *SCN1A* mutation was not identified with the 30-genes panel as it had been missed by the GS Mapper toolkit, used for data analysis. Thus, of the 176 patients analyzed with the 95-genes panel, 31 (31/176: 17.6%) carried pathogenic mutations.

Sub-analysis according to age at seizure onset of the 71 patients carrying pathogenic mutations showed that in 66% (47/71) seizures had started before the 6th month of life (Fig. 1A).

Thirty epilepsy genes were found to carry mutations at least once, ranging from nine mutations occurring in a single gene to single mutations in 17 different genes (Fig. 1B). Most mutations involved genes that are frequently associated with drug-resistant epilepsies including *SCN2A*, *SCN1A*, *STXBP1*, *KCNQ2*, *SCN8A*, *CDKL5*, and *MECP2* (Fig. 1B).

SCN2A: We found nine *de novo* variants involving this gene (Supp. Table S5). Eight patients had neonatal onset EE with seizures beginning within the 1st week of life, including two patients with Ohtahara syndrome and one with MMPSI. A remaining patient had a childhood onset EE with multifocal seizures and spasms presenting at 17 months.

SCN1A: We found eight variants involving this gene, six of which occurring *de novo* (Supp. Table S6). Although the variant c.568T>C [p.(Trp190Arg)] could not be tested in the proband's parents, it had already been published as disease causing [Fukuma et al., 2004]. Six patients had a phenotype consistent with DS, whereas the remaining two had drug-resistant infantile onset epilepsy whose clinical and EEG features were not reminiscent of a clearly defined syndrome.

KCNQ2: We found six *de novo* variants in this gene (Supp. Table S7). Four patients exhibited a neonatal onset EE, including one with Ohtahara syndrome. The remaining two probands manifested an early infantile epileptic encephalopathy (EIEE) with drug-resistant polymorphic seizures appearing 9 and 16 days after birth.

STXBP1: We found six *de novo* variants involving this gene (Supp. Table S8). Four patients exhibited a neonatal onset EE including one with Ohtahara syndrome, whereas the remaining two patients exhibited an EIEE with seizures occurring at 4 months and the last patient manifested a drug-resistant epilepsy with multifocal seizure at 12 months. Among the *STXBP1* variants, we identified a *de novo* intragenic duplication of 17.5 kb, spanning from exon 4 to exon 15 (Supp. Fig. S1). This is the first large duplication reported to involve *STXBP1*. The patient's phenotype was similar to that described with partial deletions of the same gene [Saitsu et al., 2008, Milh et al., 2011]. *In silico* prediction analysis revealed the in-frame duplication not to cause a premature truncation of the protein, despite the introduction of 371 additional aminoacids. However, we cannot exclude that the rearrangement might lead to mRNA destabilization with consequent absence of protein product or to a misfolding of the mutant protein.

SCN8A: We found five variants involving this gene, four of which occurring *de novo* (Supp. Table S9). The c.5630A>G [p.(Asn1877Ser)] variant (reported as disease causing by Anand et al., 2016) was inherited from an affected mother whose phenotype was comparable to that of her daughter. The phenotype associated with the inherited mutation observed in both the proband and her mother was mild with moderate cognitive impairment and infantile onset focal seizures persisting throughout life with a monthly frequency. Four patients exhibited an EIEE with seizures onset between 3 and 4 months.

CDKL5: We found four *de novo* variants, in two girls and two boys, involving this gene (Supp. Table S10). The c.1247_1248del [p.(Glu416Valfs∗2)] (already reported as disease causing by Raymond et al., 2013) was a mosaic mutation occurring in a hemizygous boy. Patients had an EIEE with a mean age at onset of 2 months (ranging from 1 to 3 months), the phenotype was very severe including West syndrome in two2 patients.

MECP2: We found four *de novo* variants in this gene (Supp. Table S11), all exhibiting a phenotype consistent with Rett syndrome, associated with drug-resistant epilepsy, with seizures onset ranging from 22 months to 5 years (onset at a mean age of 3 years). The patient carrying the c.915G>T [p.(Lys305Asn)] missense mutation exhibited drug-resistant focal, nocturnal seizures in childhood. During adolescence the clinical picture progressed to a phenotype resembling the PPM-X: intellectual disability with parkinsonism, pyramidal signs, and neuropsychiatric symptoms, described in males [Lindsay et al., 1996]. Among the *MECP2* variants, we identified a *de novo* intragenic deletion of 4.1 kb, involving the whole exon 3 and part of exon 4 (Supp. Fig. S1).

Rare Mutations

KCNT1, *UBE3A*, *SPTAN1*, *SYNGAP1*, *HCN1*, and *GABRB3* genes were found to carry mutations in two patients each (Supp. Table S12). All variants were demonstrated to be *de novo* with the exception of the c.1546A>G [p.(Met516Val)] variant in the *KCNT1* gene for which parental DNA was not available for testing. We classified this variant as "likely pathogenic" since it was previously reported to be disease causing by Rizzo et al. (2016).

The phenotypes of patients carrying mutations in these genes were typical, including MMPSI associated with *KCNT1* mutations [Barcia et al., 2012] and Angelman syndrome associated with *UBE3A* mutations [Sadikovic et al., 2014]. Of the two probands with *SPTAN1* mutations, one had a severe EIEE with suppression-burst EEG pattern and MRI findings of pontocerebellar and corpus callosum atrophy; the other patient manifested a less severe phenotype with drug-resistant focal epilepsy, mild cognitive impairment and Attention-Deficit/Hyperactivity Disorder. Both phenotypes resemble those already described in the literature [Tohyama et al., 2015]. Likewise, the two probands with *SYNGAP1* mutations exhibited different clinical disorders, one patient manifested early and progressive developmental delay with prominent autistic features with self-directed aggressive behavior and childhood-onset drug-resistant generalized epilepsy [Mignot et al., 2016], whereas the second patient had West syndrome since the 4th month of life. Two patients carried *HCN1* mutations, associated, in one, to a DS-like phenotype consisting of febrile and afebrile seizures since the age of 5 months, as previously described [Nava et al., 2014], whereas the second patient exhibited a catastrophic neonatal onset EE with almost continuous multifocal seizures, with prominent autonomic semiology, including prolonged apnea and severe cyanosis. He died at 14 months due to cardiopulmonary failure. The last two patients harbored *GABRB3* mutations and exhibited EIEE with polymorphic drug-resistant seizures and West syndrome.

Single variants were identified in *KCNB1*, *IQSEQ2*, *GABRG2*, *GABRA1*, *ARX*, *PCDH19*, *SLC25A22*, *MEF2C*, *CNTNAP2*, *PNPO*, *DEPDC5*, *PDHA1*, *PIGA*, *GNAO1*, *KCNA1*, *ATP1A2*, and *KCNA2*. The segregation of variants identified in the *PCDH19*, *GABRG2*, and *SLC25A22* genes could not be performed since parental DNA was not available. However, these variants were classified as "likely pathogenic" since the patients' phenotype was consistent with previous descriptions of patients carrying mutations in each of these genes. The clinical features of patients carrying mutations in these 17 genes are summarized in Table 1.

Fifteen variants (15 out of 349: 4.3%) were classified as VOUS [Richards et al., 2015] (Supp. Table S13).

Discussion

Targeted resequencing in 349 patients with pediatric drug-resistant epilepsies analyzed with two epilepsy gene panels detected 71 pathogenic mutations, 42 of which novel, in 30 different genes in 20.3% of probands. This diagnostic yield compares to previous NGS studies in epilepsy, in which rates of disease-causing variants range from 10% to 22.6% [Carvill et al., 2013; Kodera et al., 2013;

Table 1. Genes with Mutations in One Patient Each

Patient	Gender	Age	Phenotype	Age at Seizure Onset	NGS Panel	Gene	Mutation	Type	Inheritance	Reference	ExAC Database Version 0.3.1 Alternate Allele Count /Total Allele Number; Allele Frequency)	Meta SVM/ MetaLR (Liu et al., 2016)	ACMG Criteria (Richards et al., 2015)	ACMG Classification
353R	F	6 years	NOEE	18 days	95 genes	*KCNA1*	NM_000217.2:c.1214C>T p.(Pro405Leu)	Missense	*De novo*	Not reported	Not present	D/D	PS2, PM2, PP2, PP3	LP
2065P	F	4 years	Drug-resistant epilepsy with febrile and afebrile seizures	16 months	95 genes	*KCNA2*	NM_004974.3:c.971G>C p.(Ser324Thr)	Missense	*De novo*	Not reported	Not present	D/D	PS2, PM2, PP2, PP3	LP
1539P	M	6 years	West syndrome, autism spectrum disorder	9 months	95 genes	*KCNB1*	NM_004975.2:c.1109G>A p.(Trp370X)	Nonsense	*De novo*	Not reported	Not present	NA	PVS1, PS2, PM2	P
384N	M	7 years	IOEE	7 months	95 genes	*GABRA1*	NM_000806.5:c.436C>A p.(Leu146Met)	Missense	*De novo*	Johannesen et al., 2016*	Not present	D/D	PS2, PM2, PP2, PP3	LP
591P	N	6 years	Generalized epilepsy with myoclonic atonic-seizures, cognitive impairment, behavioral disorder	3 years	95 genes	*GABRG2*	NM_000816.3:c.821A>G p.(Tyr274Cys)	Missense	Parents not available	Not reported	Not present	D/D	PM2, PM6, PP2, PP3	LP
1098M	F	6 years	Childhood EE with autism spectrum disorder, microcephaly	19 months	95 genes	*IQSEC2*	NM_001111125.2:c.4039dupG p.(Ala1347Glyfs*40)	Frameshift	*De novo*	Not reported	Not present	NA	PVS1, PS2, PM2	P
1221P	F	18 years	Drug-resistant focal epilepsy with clusters of focal febrile and afebrile seizures	2 years	95 genes	*PCDH19*	NM_001184880.1:c.1339A>C p.(Asn447His)	Missense	Parents not available	Not reported	Not present	D/D	PM2, PM6, PP2, PP3	LP
174R	M	2 years	Ohtahara syndrome	1 month	30 genes	*SLC25A22*	NM_024698.5:c.394C>T p.(Gln132X)	Nonsense	Parents not available	Not reported	Not present	NA	PVS1, PM2	LP
295M	M	8 years	IOEE with autism spectrum disorder	12 months	30 genes	*MEF2C*	NM_002397.4:c.108C>A p.(Ser36Arg)	Missense	*De novo*	Not reported	Not present	D/D	PS2, PM2, PP2	LP
781M	M	6 years	Drug-resistant focal epilepsy, severe cognitive impairment	16 months	30 genes	*CNTNAP2*	NM_014141.5:c.1777+2T>C	Splice site	Homozygous	Not reported	Not present	NA	PVS1, PM2, PM3	P
543N	M	4 years	IOEE, corpus callosum hypoplasia, simplified gyral pattern	4 months	30 genes	*ARX*	NM_139058.2:c.1058C>T p.(Pro353Leu)	Missense	*De novo*	Stromme et al., 2002	Not present	D/D	PS1, PS2, PM2, PM5, PP2, PP3	P
278N	M	9 years	NOEE	1 day	30 genes	*PNPO*	NM_018129.3:c.674G>A p.(Arg225His)	Missense	Homozygous	Plecko et al., 2014	7/121398; 0.00005766	D/D	PS1, PS3, PM2,PM3, PP2, PP3	P
261H	F	20 years	Drug-resistant focal epilepsy	2 years	95 genes	*DEPDC5*	NM_001242896.1: c.3230_3234del p.(Ala1077Aspfs*82)	Frameshift	Maternally inherited	Not reported	Not present	NA	PVS1, PM2, PM4, PP1, PP4	P
1414P	F	6 years	IOEE, microcephaly, brain atrophy, partial corpus callosum agenesis	9 months	95 genes	*PDHA1*	NM_000284.3:c.904C>T p.(Arg302Cys)	Missense	*De novo*	Dahl et al., 1992	Not present	D/D	PS1, PS2, PS3, PM1, PM2, PM5, PP3	P

(*Continued*)

Table 1. Continued

Patient	Gender	Age	Phenotype	Age at Seizure Onset	NGS Panel	Gene	Mutation	Type	Inheritance	Reference	ExAC Database Version 0.3.1 Alternate Allele Count /Total Allele Number; Allele Frequency)	Meta SVM/ MetaLR (Liu et al., 2016)	ACMG Criteria (Richards et al., 2015)	ACMG Classification
1639R	M	2 years	IOEE, movement disorder with dyskinesias, microcephaly	6 months	95 genes	*PIGA*	NM_002641.3:c.404C>T p.(Ala135Val)	Missense	*De novo*	Not reported	Not present	T/T	PS2, PM2, BP4	LP
281P	M	6 years	Drug-resistant focal epilepsy, severe cognitive impairment, movement disorder with dyskinesias, microcephaly	8 months	95 genes	*GNAO1*	NM_020988.2:c.625C>T p.(Arg209Cys)	Missense	*De novo*	Saitsu et al., 2016	Not present	D/D	PS2, PM1, PM2, PP2, PP3	P
413N	M	4 years	EIEE	1 month	30 genes	*ATP1A2*	NM_000702.3:c.1097G>C p.(Gly366Ala)	Missense	*De novo*	Not reported	Not present	D/D	PS2, PM2, PP2, PP3	LP

M, male; F, female; EE, epileptic encephalopathy; NOEE, Neonatal Onset Epileptic Encephalopathy; IOEE, Infantile Onset Epileptic Encephalopathy; D, Deleterious; T, Tolerated; P, Pathogenic; LP, Likely pathogenic; NA, Not applicable; *, same patient.

Wang et al., 2014; Mercimek-Mahmutoglu et al., 2015; Trump et al., 2016]. Patients selection for gene panel analysis might influence the mutation rate. The proportion of mutations identified in our cohort might be slightly underestimated especially for the *SCN1A*, *SLC2A1*, and *PCDH19* genes since their pathogenic variants are usually associated to relatively specific phenotypes, often prompting direct sequencing as single genes. Indeed, in the same time frame of our study (2013–2015), guided by clinical features directing to distinctive phenotypes, through Sanger sequencing, we identified *SCN1A* mutations in 73 patients, *SLC2A1* mutations in 16 and *PCDH19* mutations in 13. There is one NGS study with a diagnostic yield reaching nearly 50% in which, however, the analysis was performed in a small cohort of patients [Lemke et al., 2012]. This proportion has remained much higher than subsequently reported, likely due to both the large number of targeted genes (265) and selection bias toward probands with distinctive phenotypes, such as for example DS, ceroid lipofuscinosis, and periventricular nodular heterotopia [Lemke et al., 2012].

At first sight, the sub-analysis and comparison of the two NGS panels used in this study (30 vs. 95 genes) indicates similar mutation-detection rates. Re-analysis of a small cohort of probands who were mutation-negative after the 30-genes panel screening, uncovered "pathogenic" mutations only in five additional patients, one of which involving a gene already included in the first panel. However, if all 349 patients had been analyzed with the 30-genes panel, we would have obtained only 49 mutations corresponding to 14.0% (49/349) diagnostic yield. Otherwise, if only the 95-genes panel were used, it would have identified all 71 mutations, leading to a detection-rate equal to, or likely higher than, 20.3% (71/349). Thus, the expansion of the panel from 30 to 95 genes brought to an increase of about 25% in the diagnostic yield, providing a diagnosis that would have otherwise been missed in 22 additional patients (22/349: 6.3%).

Clinical evaluation of patients carrying pathogenic variants shows that 66% of them had their first seizure prior to 6 months of age (Fig. 1A). This finding confirms that current NGS analysis leads to the highest diagnostic yield in patients with early infantile onset epilepsies [Trump et al., 2016].

Among the 71 disease-causing variants, 42 (59.2%) involved genes that are usually associated to EIEE (Supp. Tables S5–S11) including *SCN2A* (nine mutations, 12.9%) with the higher number of pathogenic variants followed by *SCN1A* (eight mutations, 11.4%), *KCNQ2* and *STXBP1* (six mutations, 8.6%), *SCN8A* (five mutations, 7.1%), *CDKL5* and *MECP2* (four mutations, 5.7%) (Fig. 1B). Besides *SCN1A*, for which the rate of mutation positive patients is undersized in our series, due to this gene being often individually screened, for the remaining genes, our findings show that in a heterogeneous cohort of pediatric drug-resistant epilepsies the possibility of harboring mutations in other genes reaches 2.6% for *SCN2A*, 1.7% for both *KCNQ2* and *STXBP1*, 1.4% for *SCN8A*, and 1.1% for *CDKL5* or *MECP2*. NGS of cohorts of patients with an age at seizure onset of less than 6 months might lead to higher percentages for each of these genes.

Although *SCN8A* mutations associated with severe EIEE are *de novo* [Larsen et al., 2015] recently, a few familial cases carrying dominant mutations have been reported in association with benign familial infantile seizures and paroxysmal dyskinesia [Gardella et al., 2016]. In our cohort, one of the five *SCN8A* mutations had familial distribution, segregating in the proband and in her affected mother, both exhibiting moderate cognitive impairment and infantile onset focal seizures persisting throughout life with a monthly frequency. The same mutation has been reported in a small family with early onset focal seizures and no cognitive impairment [Anand et al., 2016].

Twenty-nine disease-causing mutations (40.8%) were identified in 23 additional genes (Table 1, Supp. Table S12, and Fig. 1B), most of them recently associated with epilepsy and thus with phenotypes not fully characterized.

A subset of patients included in our cohort (Supp. Table S3) had undergone individual genes screening prior to being analyzed using one or both gene panels and two pathogenic variants, one in *SCN1A* and one in *UBE3A*, identified using the panels were undetected by Sanger sequencing, confirming that NGS has the potential of detecting previously missed mutation negative DS and Angelman syndrome patients. For example, Djémié et al. (2016) reported *SCN1A* mutations identified with NGS but initially missed using Sanger sequencing.

An added value of medium size NGS panels, including the one used in our laboratory targeting 95 epilepsy genes, is the detection of mutations in genes accounting for rare disorders that are usually not studied in diagnostic settings. Detection of novel mutation-positive patients allows broadening the clinical phenotype and better delineating the electroclinical features of seizures related to mutations in a given gene. For instance, mutations in the *HCN1* gene were recently associated to a phenotype resembling DS in six probands [Nava et al., 2014]. We identified two *HCN1* novel missense mutations confirming the DS like phenotype in one proband but expanding the phenotype to a catastrophic neonatal onset EE with almost continuous multifocal seizures leading to early death in one child. Likewise, mutations in *SYNGAP1* were reported in patients with early onset developmental delay followed by autistic features and childhood onset refractory generalized epilepsy with atypical absences or myoclonic-atonic seizures [Mignot et al., 2016]. We identified two mutations in this gene associated with a phenotype overlapping previous descriptions in one patient, whereas the other patient exhibited West syndrome. Therefore, *SYNGAP1* can be added to the list of genes that might cause infantile spasms. A single mutation was identified in the *ATP1A2* gene in a child with severe EE with seizures onset in the 1st month of life. Mutations in this gene are usually associated with familial hemiplegic migraine that rarely co-occurs with seizures [Bianchin et al., 2010]. *ATP1A2,* together with *ATP1A3,* belongs to a family of genes coding for catalytic subunits of Na/K-ATPase. The relevance of these genes in genetic epilepsies is also supported by the report of a child with catastrophic early life epilepsy and shortened survival carrying a mutation in *ATP1A3* [Paciorkowski et al., 2015].

Single mutations were identified in *GNAO1* and *PIGA* genes, both associated to EIEE including Ohtahara syndrome and migrating partial seizures [Nakamura et al., 2013, Kato et al., 2014; Saitsu et al., 2016]; mutations of *GNAO1* also cause a severe movement disorder [Ananth et al., 2016; Saitsu et al., 2016]. Our findings confirm the association of severe early onset epilepsy and movement disorder in the proband with the *GNAO1* pathogenic variant, yet a prominent dystonic/dyskinetic movement disorder was also observed in the proband carrying *PIGA* mutation.

Genes related to sodium channel function including *SCN1A, SCN2A, SCN8A, SCN9A,* and *SCN1B* have long been known to be associated with epilepsy of variable severity [Hildebrand et al., 2013; Brunklaus et al., 2014]. In our patients, sodium channelopathies accounted for 6.3% of the total cohort (22/349) and for about one-third of mutated genes (22/70: 31.4%). Likewise, potassium channelopathies are emerging as a major cause of EE and the list of voltage-gated potassium channel genes associated with epilepsy is growing to include *KCNB1* [Torkamani et al., 2014; Saitsu et al., 2015; Allen et al., 2016], *KCNA1* [Eunson et al., 2000] and *KCNA2* [Syrbe et al., 2015], in addition to the initial reports on *KCNT1* [Barcia et al., 2012] and *KCNQ2* [Weckhuysen et al., 2012; Kato et al., 2013]. In our series, potassium channelopathies accounted for 3.1% of the total cohort (11/349) and about 15% of mutated genes (11/70: 15.7%). Among them, using the 95-genes panel, we uncovered mutations in these genes in three patients whose phenotype was concordant with previously published probands for *KCNQ2* and *KCNT1*, whereas the child harboring the *KCNB1* mutation exhibited West syndrome. There are only seven patients reported to carry missense mutations in this gene and three of them presented infantile spasms [Torkamani et al., 2014, Saitsu et al., 2015; Allen et al., 2016]. The patient carrying the *KCNA2* variant exhibited late infantile drug-resistant seizures followed by a good outcome with seizure-freedom in childhood, confirming the spectrum of *KCNA2*-related phenotypes from early and severe infantile EE to mild epilepsy with good outcome [Syrbe et al., 2015]. The severity of the phenotypes appears to correlate with the genotype: gain-of-function mutations are more severe with persistent seizures. In contrast, patients with loss-of-function mutations have later seizure onset, and achieve seizure freedom in childhood [Syrbe et al., 2015]. We hypothesize that our patient belongs to this latter group. Our 95-genes panel included the *KCNA1* gene, which has previously been associated to episodic ataxia/myokymia syndrome in humans [Browne et al., 1994] and to limbic seizure-phenotype similar to temporal lobe epilepsy in Kcna1-null mice [Robbins et al., 2012]. We identified a *de novo KCNA1* variant in a patient with refractory neonatal onset focal seizures whose semiology, as the child grew older, progressed to facial fearful expression with automatisms mimicking hold and reassurance seeking. Such semiology is suggestive of a limbic origin of seizures, as also observed in the mouse model. Thus, the gene function, *de novo* occurrence, and the similarity with the mouse model support a causative role for the *KCNA1* variant.

Array-CGH was only prformed in one third of our patients since the diagnostic yield of this approach is considered to be low in patients with a pure EE phenotype (about 3%) [Epilepsy Phenome/Genome Project Epi4K Consortium, 2016]. Array-CGH reaches instead higher percentages of positive findings when epilepsies co-occur with additional findings such as abnormal MRI, developmental delay, or dysmorphic features [Mefford, 2015]. Yet, patients with drug-resistant epilepsy and no additional features are more likely to carry intragenic rearrangements that might be uncovered by methods including exon-level microarray, which represent a useful complement to gene panel analysis. Indeed, using the GS Mapper tool and the long reads generated by the 454 pyrosequencing of the 30-genes panel, we identified one large duplication in *STXBP1* and one large deletion in *MECP2*.

NGS panels, now used widely in clinical settings to identify genetic causes of epilepsy, have greatly improved and expedited the diagnostic approach to patients with intractable epilepsy. The results of such panels help the diagnostic process and solve complex and puzzling cases. They also help clinicians improving management, avoiding numerous investigations including invasive procedures, prognostication, guiding treatment choices in some cases, and providing appropriate genetic counseling to families.

Considering the rapid advances in the area of epilepsy genetics, a gene panel might soon become obsolete. In both our panels, genes that were considered pathogenic at the time of the panel design and now reclassified as susceptibility loci (i.e., *CLCN2*, *EFHC1*) or as loci of conflicting interpretation (i.e., *PRICKLE2*) should be removed. However, genes that were not recognized as disease causing at the time of the panel design (i.e., *ALG13*, *SLC1A2*) were not included. For this reason, a continuous revision of a gene panel is advised, through redesign and revalidation before its implementation in the molecular diagnosis process. An updated version of the currently

gene panel used in our Neurogenetics Laboratory has been included in the supplementary material (Supp. Table 14S).

In conclusion, larger and more comprehensive panels may be indicated when the phenotype is not specific of well-defined epilepsy syndromes, as they enable molecular diagnosis of atypical phenotypes, thus allowing to broaden phenotype–genotype correlations. In the diagnostic setting, carefully and frequently updated gene panels targeting about 100 epilepsy genes allow a good sequence coverage of each gene, requiring manageable bioinformatic analysis, with affordable costs. These panels have become cheaper while turnaround time for results is also decreasing. The cost of a gene panel analysis in a diagnostic setting is now similar to that of Sanger sequencing for a single gene. Therefore, while targeted single-gene Sanger sequencing may remain appropriate in some cases, for example, typical *SCN1A*-related phenotypes or classical Rett syndrome (*MECP2*) or *GLUT1* deficiency syndrome with hypoglycorrhachia, our data strongly support the use of panel-based analysis as the diagnostic genetic test of choice in the majority of individuals with intractable early-onset seizure.

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