Targeted next generation sequencing as a diagnostic tool in epileptic disorders

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SUMMARY

Purpose: Epilepsies have a highly heterogeneous background with a strong genetic contribution. The variety of unidentifyable and overlapping syndromic and nonsyndromic phenotypes often hampers a clear clinical diagnosis and prevents straightforward genetic testing. Knowing the genetic basis of a patient’s epilepsy can be valuable not only for diagnosis but also for guiding treatment and estimating recurrence risks.

Methods: To overcome these diagnostic restrictions, we composed a panel of genes for Next Generation Sequencing containing the most relevant epilepsy genes and covering the most relevant epilepsy phenotypes known so far. With this method, 265 genes were analyzed per patient in a single step. We evaluated this panel on a pilot cohort of 33 index patients with concise epilepsy phenotypes or with a severe but unidentifyable seizure disorder covering both sporadic and familial cases.


Significance: We have developed a fast and cost-efficient diagnostic screening method to analyze the genetic basis of epilepsies. We were able to detect mutations in patients with clear and with unidentifyable epilepsy phenotypes, to uncover the genetic basis of many so far unresolved cases with epilepsy including mutation detection in cases in which previous conventional methods yielded falsely negative results. Our approach thus proved to be a powerful diagnostic tool that may contribute to collecting information on both common and unidentifyable epilepsy disorders and in delineating associated phenotypes of less frequently mutated genes.

KEY WORDS: NGS epilepsy panel, Mutation screening, Genetic diagnostics, Massive parallel sequencing.

Epilepsy is a common and highly heterogeneous neurologic disorder. Especially the pediatric epilepsies have a very diverse background, and seizures may often represent only one aspect of an underlying complex syndrome (Muthugovindan & Hartman, 2010).
Among hereditary factors known to account for epilepsy, all kinds of genetic aberrations have been described, for example, monogenic, complex, mitochondrial, chromosomal, and imprinting. (Claes et al., 2001; Weber & Lerche, 2008; Baulac & Baulac, 2010; Klassen et al., 2011; Mulley & Mefford, 2011) However, in the majority of cases seen in clinical practice the underlying genetic aberrations remain elusive. This may, of course, be due to unidentified epilepsy genes as well as complex inheritance of a subset of pathogenic or predisposing factors. However, it seems plausible that numerous patients with an epilepsy phenotype not suggestive of a specific genetic disorder may still have aberrations in known but rarely mutated genes.

Identifying the genetic background of a patient’s epilepsy phenotype may help in counseling the patient and relatives concerning a possibly elevated recurrence risk. The definitive diagnosis is important to avoid an often endless series of laborious, stressful, and expensive diagnostic procedures. In some cases it may also allow the prediction of future development or even guide antiepileptic pharmacotherapy, as for example by starting a ketogenic diet in glucose transporter type 1 (Glut1) deficiency or avoiding administration of Na+ channel blockers in Dravet syndrome (Brodie et al., 2011). Furthermore, detecting mutations in known epilepsy genes in patients with an uncommon or unspecific presentation of a seizure disorder may help to reveal the true phenotypic spectrum of the disorder.

Establishing a precise clinical diagnosis of a genetic disease with an unspecific epilepsy phenotype is a challenging task. Additional features such as accompanying disorders, patient and family history, laboratory parameters, dysmorphism, or malformation may help in finding the diagnosis only if they are specific. Screening for underlying genetic defects is often complicated by the multitude of putatively responsible genes, each with a low individual prevalence and mutation detection rate.

Mutation screening with conventional Sanger sequencing is considered the “gold standard” with respect to sequence quality. However, this method allows screening of only one gene at a time, exon by exon. In contrast, recently developed Next Generation Sequencing (NGS) techniques allow massive parallel sequencing of as many genes as desired. Here, the sequence quality depends on the minimum coverage at a base-pair position and the optimization of enrichment procedures of the genes of interest (target sequence) (Mamanova et al., 2010; Metzker, 2010). Targeted NGS seems particularly appropriate to screen for mutations in disorders with a highly heterogeneous genetic background, such as cardiomyopathies, neuromuscular diseases, retinopathies, and not least, epilepsies.

The power in detecting mutations in positive controls using an NGS panel of genes for ataxia and for congenital disorders of glycosylation has recently been shown (Hoischen et al., 2010; Jones et al., 2011). Shearer et al. (2010) successfully tested an NGS panel containing 54 genes related to hearing loss and revealed mutations in five of six patients.

We developed an NGS panel of genes comprising the most relevant epilepsy genes and epilepsy phenotypes (n = 265 genes). We screened a pilot cohort of 33 index patients representing both sporadic and familial cases with either concise epilepsy phenotypes or severe and often unspecific epileptic disorders, often with additional symptoms such as myoclonus or mental retardation. We aim to demonstrate how this technique may facilitate and accelerate the detection of the underlying genetic defect of a patient’s epilepsy syndrome.

**Materials and Methods**

**Patients**

We collected a cohort of 33 index cases with very different epilepsy phenotypes from various hospitals and clinical practices in Germany and Switzerland. The patients were selected randomly. A few patients had been screened previously for mutations in selected genes with conventional techniques. However, none of the patients had hitherto known genetic aberrations.

Written informed consent for genetic testing according to the respective national law was obtained from all tested individuals or their respective legal guardians prior to analysis.

**Epilepsy panel**

We chose 265 genes that were known to be involved in monogenic disorders including epilepsy as a phenotypic feature according to the Online Mendelian Inheritance in Man (OMIM) database (http://www.ncbi.nlm.nih.gov/omim). This panel of genes covers exonic regions as well as exon–intron boundaries of most of currently known epilepsy genes.

We used a customized target in solution enrichment followed by Next Generation Sequencing to screen for mutations in the 265 candidate genes (875,678 bp in total). Briefly, we fragmented the human genome, enriched for the coding regions of genes by using complementary highly specific RNA baits (Agilent Sure Select Target Enrichment, Agilent, Santa Clara, CA, U.S.A.) and then sequenced consecutively on a SOLiD 4 platform (Life Technologies, Carlsbad, CA, U.S.A.). We sequenced two patients on 1/8 (1 Oct) of a full slide. In total we sequenced 33 patients on 16.5 Octs. The hereby-generated 50 bp reads were mapped to the human genome and analyzed using the BioScope software versions 1.2. (Life Technologies). BioScope processes the reads with a Blast-like mapping algorithm using color codes. The single nucleotide variant (SNV) calling is implemented with a frequentist algorithm on high coverage positions or a Bayesian algorithm. Both algorithms fully support dicoles.

The detected variants were evaluated for their context to the individual phenotype (according to OMIM). All
putatively causative mutations (Table 2) were validated by conventional Sanger sequencing. To further determine the impact of novel variants, we tested for segregation in families if possible. In all cases, we aimed to have complete SNV coverage of all genes relevant for the patient’s phenotype. Hence, we resequenced all underrepresented regions (coverage ≤10 SOLiD reads) by Sanger in all relevant genes.

We tried to estimate the putative pathogenic effect of every suspected mutation with conventional and freely available online tools, such as Polyphen2 (http://genetics.bwh.harvard.edu/pph2/), MutationTaster (http://www.mutationtaster.org/MutationTaster/documentation.html) and Human Splicing Finder (http://www.umd.be/HSF/) (Desmet et al., 2009; Adzhubei et al., 2010; Schwarz et al., 2010).

**Results**

**Patients**

We sequenced the DNA of 33 patients with different epileptic syndromes (Table 2). Eight of these 33 patients had a well-defined phenotype, as for example Dravet syndrome,
Table 2. Patients analyzed with the NGS epilepsy panel with an overview over the individual patient data (left), the amount of detected variants in each patient (middle) as well as the mutations being detected, their validation and refinement of diagnosis (right).

<table>
<thead>
<tr>
<th>Subcohort/case</th>
<th>Sex</th>
<th>Age in years</th>
<th>Suggested clinical diagnosis</th>
<th>Symptoms of index patient</th>
<th>Symptoms of relatives</th>
<th>Variants on target</th>
<th>Novel to dbSNP131</th>
<th>Novel to dbCaGt</th>
<th>Mutations cDNA (detected by NGS and confirmed by Sanger sequencing)</th>
<th>Mutations protein</th>
<th>Mutation known to literature</th>
<th>Segregation with phenotype</th>
<th>Final diagnosis</th>
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<td>Subcohort/case</td>
<td>Sex</td>
<td>Age in years</td>
<td>Suggested clinical diagnosis</td>
<td>Symptoms of patients</td>
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<td>Variants on target</td>
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<td>B.255</td>
<td>m</td>
<td>7</td>
<td>Doose</td>
<td>GTCS, myoclonus, DD</td>
<td>No</td>
<td>253</td>
<td>116</td>
<td>22</td>
<td>SCNN1G1 c.833A&gt;G/ c.1266G&gt;C</td>
<td>TPP1 p.Q278R/ p.Q422H SCN1A p.R222X</td>
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<td>Yes</td>
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<td>B.563</td>
<td>m</td>
<td>5</td>
<td>Dravet-like</td>
<td>Myoclonic atatic seizures, DD</td>
<td>Brother (similarly affected)</td>
<td>552</td>
<td>281</td>
<td>161</td>
<td>SCN1A c.664C&gt;T</td>
<td>Yes</td>
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<td></td>
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<td>B.202</td>
<td>m</td>
<td>22</td>
<td>Dravet</td>
<td>GTCS, myoclonus, FS, DD</td>
<td>No</td>
<td>640</td>
<td>276</td>
<td>172</td>
<td>No/yes</td>
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<td>f</td>
<td>33</td>
<td>Unclear</td>
<td>GTCS, progressive EE, ataxia, spasticity, DD</td>
<td>No</td>
<td>298</td>
<td>127</td>
<td>26</td>
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<td>Yes</td>
<td></td>
<td></td>
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<tr>
<td>C.211</td>
<td>m</td>
<td>33</td>
<td>Unclear</td>
<td>GTCS, ataxia, spasticity, DD</td>
<td>Sister (similarly affected)</td>
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<td>121</td>
<td>27</td>
<td>KCNJ10 c.203T&gt;C/ c.385A&gt;G</td>
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<td>No</td>
<td>Yes</td>
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<td>f</td>
<td>15</td>
<td>Unclear</td>
<td>GTCS, DD, dygenesis of left hippocampus</td>
<td>No</td>
<td>296</td>
<td>135</td>
<td>19</td>
<td>No/en</td>
<td></td>
<td></td>
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<td>m</td>
<td>30</td>
<td>Partial epilepsy &amp; DD</td>
<td>GTCS, absences, partial seizures, myoclonus, DD</td>
<td>No</td>
<td>350</td>
<td>140</td>
<td>25</td>
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<tr>
<td>C.274</td>
<td>m</td>
<td>32</td>
<td>Unclear</td>
<td>Myoclonic epilepsy</td>
<td>No</td>
<td>491</td>
<td>211</td>
<td>88</td>
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<td>f</td>
<td>5</td>
<td>Unclear</td>
<td>GTCS, DD, behavioral disorder</td>
<td>No</td>
<td>467</td>
<td>182</td>
<td>59</td>
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<td>17</td>
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<td>No</td>
<td>296</td>
<td>119</td>
<td>24</td>
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<td>C.345</td>
<td>m</td>
<td>28</td>
<td>Unclear</td>
<td>GTCS in infancy</td>
<td>No</td>
<td>279</td>
<td>129</td>
<td>41</td>
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<td>2</td>
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<td>GTCS, RSE, microcephaly, DD, frontal polymicrogyria</td>
<td>No</td>
<td>209</td>
<td>89</td>
<td>20</td>
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<td>No/no</td>
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<td>C.517</td>
<td>m</td>
<td>6</td>
<td>Unclear</td>
<td>GTCS, myoclonus, ataxia, hysparhythmia, mild DD</td>
<td>No</td>
<td>228</td>
<td>98</td>
<td>24</td>
<td>SCNN1G1 c.639A&gt;T</td>
<td>SCNN1A p.231P</td>
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<td>dh</td>
<td>Atypical Dravet</td>
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<td>8</td>
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<td>GTCS, myoclonus, hypotonia, spasticity, DD</td>
<td>No</td>
<td>258</td>
<td>103</td>
<td>19</td>
<td>SCNN1G1 c.639A&gt;T</td>
<td>SCNN1A p.231P</td>
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<td>C.524</td>
<td>m</td>
<td>23</td>
<td>IGE</td>
<td>Sporadic GTCS (onset 20 years), Brugada</td>
<td>No</td>
<td>235</td>
<td>111</td>
<td>23</td>
<td>Brothet, father (rare GTCS), uncle, granduncle (SUDEP)</td>
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<td>38</td>
<td>IGE</td>
<td>GTCS, dissociative disorder</td>
<td>No</td>
<td>470</td>
<td>224</td>
<td>127</td>
<td>Brugada</td>
<td>Yes</td>
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<tr>
<td>C.555</td>
<td>m</td>
<td>10</td>
<td>Unclear</td>
<td>Partial epilepsy, DD, subependymal heteroepia</td>
<td>No</td>
<td>473</td>
<td>232</td>
<td>138</td>
<td>SCNN1G1 c.639A&gt;T</td>
<td>SCNN1A p.231P</td>
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Continued
ceroid lipofuscinosis (CLN), or early infantile epileptic encephalopathies (EIEE), suggesting one or more candidate genes (subcohort A: 208, 214, 218, 239, 515, 520, 561, 688). Ten of 33 patients (subcohort B: 109, 219, 231, 264, 280, 310, 521, 525, 563, 802) were assigned to phenotypes for which the genetic background is largely unknown, such as rolandic epilepsy, myoclonic–astatic epilepsy, Lennox-Gastaut syndrome, or SCN1A-negative Dravet syndrome. The remaining 15 patients (subcohort C: 210, 211, 238, 243, 274, 303, 335, 345, 514, 517, 519, 524, 526, 555, 942) had unspecific manifestations of seizures with or without mental retardation.

Table 2 provides an overview of symptoms, clinical diagnosis, and further affected family members of the 33 patients included in this study. The ages of onset were reported as available but were often unknown.

Ten patients had a positive family history regarding their individual disorder. The remaining 23 patients were sporadic cases.

Epilepsy panel

We chose 265 genes associated with epileptic seizures to create a comprehensive diagnostic tool with a diversified spectrum of application. To facilitate data analysis we further subdivided the 265 genes into 18 virtual subpanels according to clinically relevant groups of epilepsy phenotypes (Table 1). Some of the subpanels are overlapping to a certain extent due to phenotypic variability associated with certain epilepsy genes. This rough grouping of genes is not exhaustive but allows a prioritization of diagnostically important genes in an individual.

At least 35 million reads were generated per patient. Each read had a length of about 50 bp. The alignment of all reads to the human genome led to an average coverage of 292 per base pair (maximum coverage $>$2,500). About 98.4% of mapped reads were on targeted regions.

Table 2 summarizes the results of the panel analysis in all 33 patients of our cohort. We excluded all variants that were not on target and then detected on average 326 variants within the 265 panel genes per patient (range 209–640). Furthermore, we did not consider intronic variants outside of common splice regions (±30 bp) as well as synonymous exonic variations outside the highly conserved acceptor and donor splice sites. On average we identified 144 nonsynonymous exonic variants or putative splice variants per patient (range 72–288). Forty-eight of these variants (range 8–190) were novel to the dbSNP 131 (http://www.ncbi.nlm.nih.gov/projects/SNP/).

Finally, we compared the variants with our in-house database. At the time of study, this database contained the data of about 100 individuals investigated by whole-exome sequencing on the same platform. Referral reasons of these 100 individuals were deafness, neurodegenerative diseases, as well different types of cancers. With this comparison we aimed to identify sequence platform specific errors. Variants that appeared $\geq 2x$ in this database might represent...
sequencing artifacts or so far unreported single-nucleotide polymorphisms (SNPs) and their analysis was thus postponed. This led to a further decrease of the number of truly novel variants down to 15 per patient (range 3–77). However, in patients with no apparent mutation we still evaluated all variants not annotated in dbSNP independently of being annotated in our in-house database.

By the design of our panel analysis, we targeted all coding regions of the 265 genes as well as all intron–exon boundaries. However, certain regions did not achieve a satisfying coverage of ≥10 and needed resequencing by Sanger (one polymerase chain reaction [PCR] for each region) to complete SNV calling (Fig. 1B). In patients without obvious mutations, the amount of resequencing depended on the amount of genes that were considered as being relevant according to the patient’s individual phenotype. Together with the validation of putatively causative variants, this led to a resequencing effort of up to 30 PCRs per patient.

Identification of mutations

We identified disease-causing aberrations in 16 of 33 patients (48%). Table 2 gives an overview of all mutations detected in our study.

In all eight patients of subcohort A (Table 2), the previously suggested clinical diagnosis could be confirmed by detection of mutations within one of the candidate genes. This comprises two patients with classical Dravet syndrome (218, 515) having mutations in \( \text{SCN1A} \) (p.E289V and p.F1330Lfs3X), a female with biparietal nodular heterotopias (520) due to an \( \text{FLNA} \) mutation (p.R226X) as well as a family with benign familial infantile seizures (index 214) and a \( \text{SCN2A} \) mutation (p.V208E). In two patients with the heterogeneous phenotype of EIEE (208, 561), we confirmed the diagnosis by detecting the mutations p.R122X in \( \text{STXBP1} \) and p.M379R in \( \text{SCN1A} \). Furthermore, we revealed the compound heterozygous mutations p.T294K/p.A138D fs10X in \( \text{MFSD8} \) as the cause for a patient’s late-infantile CLN (688). Finally, we detected the homozygous missense mutation p.L108M in \( \text{KCTD7} \) in patient 239 with progressive myoclonus epilepsy (PME) as well as in the similarly affected sibling (Fig. 2B). Aberrations in \( \text{KCTD7} \) have been described in only one single family before (Van Bogaert et al., 2007). Our family has therefore also been included in a cohort (Kousi et al., 2012) describing several new cases with PME and homozygous or compound heterozygous mutations in \( \text{KCTD7} \) leading to the suggestion that \( \text{KCTD7} \) might contribute more frequently to PME than previously estimated.

Among the 10 patients of subcohort B with distinctive clinical phenotypes of unknown genetic cause, we detected the underlying mutations in five Patients. Those included two patients with so far “\( \text{SCN1A} \)-negative Dravet syndrome” (521, 802) analyzed by other laboratories previous to our study, in which we detected \( \text{SCN1A} \) mutations (p.R393H and p.R222X). In Patient 563 as well as in his dizygotic twin brother, both with developmental delay,
myoclonic–astatic seizures, and few clinical similarities to Dravet syndrome, we were able to detect compound heterozygosity for p.Q278R and p.Q422H in \(TPP1\) leading to the diagnosis of late infantile CLN (Fig. 2C). Consequently performed enzyme analysis as well as a magnetic resonance imaging (MRI) investigation further confirmed this diagnosis. Hence, genetic analysis was able to establish diagnosis even before and independent of the precise clinical evaluation. In a family with three adult brothers with seizures and mental retardation, we identified the hemizygous X chromosomal mutation p.R290H in \(ARHGEF9\) in the index patient (310) leading to the diagnosis of EIEE8. Unfortunately, we were not able to perform segregation analysis in this family. Finally, we detected the mutation p.P574S in \(KCNQ3\) in a patient with rolandic epilepsy and additional features, such as mild developmental delay and abnormal behavior. According to the literature, the pathologic effect of this mutation is unclear (Miceli et al., 2009). However, it has been detected in four individuals with rolandic epilepsy from two different families as well as in eight individuals in a cohort of 455 IGE patients (Neubauer et al., 2008). It was not detected in any of the 454 controls. In dbSNP, p.P574S in \(KCNQ3\) has been reported as rs74582884 in 4 of 2,188 alleles of the 1,000 Genome project (‘‘minor allele frequency/minor allele count’’ of 0.0018/4) and is interpreted as being “pathogenic.” However, in our view this has to be interpreted with caution, since the pathogenicity of this variant has not been convincingly shown so far.

Among the 15 cases of subcohort C with unspecific clinical phenotypes, we detected aberrations in three patients. In Patient 517 with generalized and myoclonic seizures (onset 14 months), hypersrrhythmic EEG pattern, mild ataxia, and mild developmental delay, we detected the heterozygous de novo mutation c.693A>T in \(SCN1A\). This aberration does not alter the amino acid sequence (p.P231P). However, it replaces a purine with a pyrimidine at the second last

Figure 2.

(A) The heterozygous mutation p.V208E in \(SCN2A\) segregates with the phenotype within the family of index Patient 214 (arrow). All affected individuals were reported to have had at least one cluster of generalized seizures during the first months of life. None had developmental delay or seizures later than 13 months of age, confirming the diagnosis of BFIS. (B) Patient 239 with progressive myoclonus epilepsy as well as his similarly affected sister represent the second family ever described with a \(KCTD7\) mutation. Both individuals were homozygous for p.L108M, whereas the healthy brother and consanguineous parents were heterozygous. (C) Both twin brothers with late-infantile ceroid lipofuscinosis were compound heterozygous for the mutations p.Q278R and p.Q422H in \(TPP1\). Both parents were heterozygous for one mutation each. (D) The unspecific syndromic phenotype of epilepsy, ataxia, and mental retardation in two similarly affected siblings was attributed to compound heterozygosity of p.L68P and p.I129V in \(KCNJ10\), leading to the very rare diagnosis of SESAME syndrome. Only the patient’s mother was available for genetic testing and appeared to be heterozygous for p.L68P in \(KCNJ10\) only.

Epilepsia © ILAE
position in exon 5. According to MutationTaster and Human Splicing Finder (Desmet et al., 2009; Adzhubei et al., 2010; Schwarz et al., 2010), this results in loss of a sequence motif at the donor splice site and is likely to disturb normal splicing. It may therefore be responsible for a clinical phenotype with overlap but still some differences to classical Dravet syndrome. Surprisingly, SCN1A mutations had previously been excluded in this patient by high-resolution melting analysis. Furthermore, in a pair of sibs with an unspecific syndromic phenotype of epilepsy, ataxia, and mental retardation (index 211) we detected the mutations p.L68P and p.I129V in KCNJ10 in a compound heterozygous state (Fig. 2D). This led to the diagnosis of SESAME syndrome (seizures, sensorineural deafness, ataxia, mental retardation, and electrolyte imbalance). Fewer than 10 families have been reported since its original description (Scholl et al., 2009). Finally, we detected a mutation in SMS predicting the amino acid substitution p.F58L in a male adult with an unspecific phenotype of generalized seizures, mental retardation, and severe osteoporosis with spontaneous fractures leading to the diagnosis of X-linked mental retardation of Snyder-Robinson type. This is again a very rare disorder, as only three families with affected male individuals have been described to date.

**Validation of mutations and segregation analysis**

All mutations have been validated and confirmed with conventional Sanger sequencing. The 16 mutation-positive cases comprise five patients (of 10/50%) with a positive family history and 11 sporadic cases (of 23/48%). In four of the five familial cases (211, 214, 239, 563), blood samples of the patient’s parents or relatives were available. Segregation of the individual mutations with the phenotype was confirmed within all four families (Fig. 2).

In three of 11 sporadic cases (218, 517, 521), parental analysis confirmed a de novo origin of the respective SCN1A mutation, as has been found in 95% of patients with Dravet syndrome (Nabbout et al., 2003). One male patient (942) had a mutation in SMS that was excluded in his mother, confirming a de novo origin of an X-linked disorder. In one further case (688), biparental inheritance could be confirmed for the compound heterozygous MFSD8 mutations.

Among the remaining six sporadic cases for whom no parental blood samples were available, three patients (264, 520, 802) were identified as carrying genetic aberrations in KCNQ3, FLNA, and SCN1A that have been already described in the literature (Claes et al., 2001; Parrini et al., 2006; Neubauer et al., 2008; Miceli et al., 2009) (Table 2). The other three (208, 515, 561) carried novel mutations in SCN1A (one nonsense, one missense) or STXBP1 (one nonsense).

In summary, the mutational spectrum comprised six known and 13 unknown mutations. Among them are two small deletions (SCN1A p.F1330Lfs3X [1 bp]; MFSD8 p.A138Dfs10X [7 bp]).

**Discussion**

**Patients**

With a mutation detection rate of 48% (16/33) our approach proved to be an efficient tool in identifying the underlying genetic defects of patients with highly varying epileptic phenotypes.

The detection of mutations in all eight individuals of subcohort A (Table 2) proves that a precise clinical phenotyping already points to specific genes. However, when a particular phenotype can be due to mutations in several genes, direct screening with conventional methods is time- and cost-consuming. For example, late-infantile CLN was found to be due to compound heterozygous mutations in MFSD8 in patient 688. In a conventional testing strategy, MFSD8 would have probably been one of the last genes screened, because mutations in TPP1, PPT1, CTSD, CLN5, and CLN6 and maybe also CLN8 are considered to be more frequent compared to MFSD8 (Mole & Williams, 2010).

We detected mutations in 5 of 10 patients of subcohort B. In these patients, conventional sequencing approaches can be very challenging due to the multitude of putatively causative genes responsible for certain phenotypes. On the other hand, many distinct epilepsy phenotypes have a widely unclear etiology and their genetic defects often remain undiscovered (as for example in Lennox–Gastaut syndrome or SCN1A-negative Dravet syndrome). Since the epilepsy panel covers a wide spectrum of differential diagnoses in epilepsy phenotypes, the detection of mutations is significantly facilitated in comparison to one by one conventional Sanger sequencing approach in these cases.

Finally, we detected mutations in 3 of 15 patients of subcohort C. In these cases, conventional sequencing approaches would most probably fail to reveal genetic aberrations as an unspecific clinical phenotype hampers the prediction of putatively causative genes. However, sequencing the epilepsy panel in this subcohort allowed the detection of mutations in patients with poor or diffuse phenotypic data.

Thus, in subcohort B, our results led to a refinement of the clinical diagnosis in many cases, whereas in subcohort C, the panel analysis was the first opportunity to enable a diagnosis in at least some of the patients.

**Epilepsy panel**

In comparison to whole exome sequencing (WES), the largely decreased number of genes within a disease-specific gene panel allows a significant increase of coverage on target sequences (Fig. 1). This helps to reliably detect disease-causing mutations as well as to prevent detection, validation, and interpretation of countless variants in genes not suspected to be related to a patient’s phenotype.

Of course, this method is only suited in diagnostics of monogenic disorders or those with at least one major gene effect. It is not suited for complex genetic disorders for
which WES as a research tool has the advantage of covering all coding genes, although with less abundant coverage.

We identified the well-known de novo mutations p.R222X and p.R393H in SCN1A (Claes et al., 2001; Ohmori et al., 2006) in two patients with classical Dravet syndrome (521, 802), although records for both patients stated negative mutation testing for SCN1A with conventional Sanger sequencing by approved laboratories in 2003 and 2009. In a third case (517), SCN1A mutations had been excluded by high-resolution melting analysis in a different laboratory. Nevertheless, we revealed the de novo putative splice mutation p.P231P in SCN1A. After detecting the mutations by the NGS epilepsy panel, we clearly confirmed their presence with Sanger sequencing in all three cases. According to one of the previous labs, the failure in detecting the p.R393H mutation was abetted by a 2 bp deletion in the adjacent intron, representing a known polymorphism that led to a misalignment of the two alleles and caused misinterpretation of the sequence data. This alignment artifact is specific to Sanger sequencing analysis and generally does not occur after mapping of data of the epilepsy panel or any other NGS approach. Unfortunately, we were not able to reconstruct the conditions that led to the failure in detecting the p.R222X and the p.P231P mutations by the other two laboratories.

In Patient 520 with bilateral periventricular heterotopia, our panel approach failed to detect a causative mutation in the first step. Investigation of FLNA, the most suspicious gene in this disorder, revealed unsatisfying coverage of several exons impeding a complete SNV calling (Fig. 1B). Subsequent Sanger sequencing of underrepresented regions revealed the known mutation p.R226X in FLNA (Parrini et al., 2006). This case illustrates that sensitivity of the panel analysis is substantially dependent on a sufficient coverage of the targeted regions.

To further improve the future diagnostic strength of the NGS epilepsy panel, we consequently optimized the coverage of genes/areas that did not perform optimally in the first version of the panel. We abandoned enrichment of regions that were out of diagnostic focus (e.g., 5' and 3' UTRs) and used the spared capacity to increase coverage of underrepresented areas. In addition, we included more genes (323 in total, Table 1) that we thought to be useful to complete the list of clinical relevant epilepsy genes compared to the first version of the panel (containing 265 genes). Preliminary data from this second version of the panel, which are not included in this paper, already show a significant improvement. Genes with gaps or drops of coverage, such as FLNA, had a far better and more even distribution of coverage and now met diagnostic requirements (Fig. 1C). This also led to a significant decrease of the amount of Sanger resequencing.

In addition to the described mutations, we also detected numerous unclassified variants in every single patient—mainly in genes that we doubt to be correlated with the patient’s individual phenotype. Some of these variants represented false positives and sequencing artifacts that were not confirmed by conventional Sanger sequencing. Others do represent known and unknown rare SNPs. At present, we were not able to validate all these variants. Especially in those cases where we were able to detect pathogenic mutations, we tended to neglect a possible but improbable major effect of these variants on the patient’s phenotype. In cases so far unresolved, the multitude of unclassified variants remains a significant problem because validation of all such variants and unequivocal exclusion of their possible pathogenic influences remain elusive at present. Hence, massive parallel sequencing of panel genes should be considered as a screening tool to detect clear monogenic or oligogenic mutations. Rare variants may contribute as a disease modifier to the genetic background of a patient’s disorder, but at present we are unable to estimate such contribution. Large cohorts and/or families and sophisticated biostatistical evaluation will be necessary to unravel this important question in genetic diseases such as familial epilepsy syndromes.

Our mutation detection algorithm focuses on variants novel to dbSNP 131. However, later versions of this database and inclusion of other sources (such as data from the 1,000 Genome project) might help to further decrease the number of truly novel variants.

For the 17 patients for whom our method failed to detect definite or putative mutations, it remains unclear whether this was due to (1) the causative gene not being among the 265 genes of the first version of the panel or not being sufficiently covered; (2) the causative mutation not being on target (e.g., both nonexonic and non–splice-site); (3) the causative mutation being detected but not identified (e.g., masked as unclassified variant) or missed (e.g., due to too stringent filtering); or (4) polygenic inheritance, chromosomal aberrations, CNVs, or other issues/problems.

In some of those 17 unresolved patients, however, we detected “novel” variants that might possibly be causative for the individual phenotypes. Due to the unavailability of parental blood samples, the validation of these variants was often exclusively restricted to in silico calculations and failed to deliver convincing evidence for a substantial confirmation of pathogenicity. However, we cannot exclude putatively causative effects for all these variants (data not shown). Still, similar to routine conventional genetic testing, the availability of parental blood samples may often facilitate the evaluation of a novel variant.

With our study, we demonstrate the efficiency of detecting genetic aberrations in patients with epilepsy using an NGS panel approach. In several cases we detected mutations in genes that were uncommonly mutated and are thus not routinely screened in conventional genetic testing strategies using Sanger sequencing. With the epilepsy panel, the detection rate of genetic aberrations no longer depends on the prerequisite of a precise clinical diagnosis and consecutive prediction of putatively casual genes. It rather seems sufficient to categorize the patient’s epilepsy in a
rough group of phenotypes (e.g., according to a subpanel) and to evaluate mainly the respective genes. With the panel approach it was possible to uncover diagnoses in cases with unspecified phenotypes or poor clinical data (e.g., 211, 310, 942). Of interest, some of the diagnoses revealed by the epilepsy panel represent extremely rare disorders (e.g., 211, 239, 310, 942). In patients with more than one gene being suspected to account for the phenotype, the epilepsy panel turned out to be superior to conventional Sanger sequencing in both processing time and analysis cost. The cost of this panel analysis will be comparable to sequencing 1–3 medium- to large-sized genes by conventional techniques.

The detection of SCN1A mutations in three apparently SCN1A-negative cases underlies the diagnostic strength of the epilepsy panel in comparison to conventional techniques. Hence, even in cases with a clear phenotype and only one gene in diagnostic focus, primary screening with the epilepsy panel is certainly worth consideration.

In summary, we feel that this newly developed disease-specific NGS panel of genes represents a powerful tool for uncovering underlying genetic defects and for establishing a definite diagnosis in patients with various epilepsy disorders, and it strongly contributes to a better understanding of genotype–phenotype correlations.

ACKNOWLEDGMENTS

This study has been partially funded by the Novartis Stiftung für Biologisch-Medizinische Forschung (10B46), by the Swiss League Against Epilepsy (Forschungsförderpreis 2010), by the Bundesministerium für Bildung und Forschung in Germany (BMBF: NGFNplus, EMNNet/01GS08123; Netzwerk Seltene Erkrankungen, InnOnetOnet/01GM1105A, TP5 and TP7) and by the European Science Foundation (Eurocores programme EuroEPINOMICS) through the Deutsche Forschungsgemeinschaft (DFG/Le1030/11-1) and the Schweizer Nationalfond (SNF 32EP30_136042/1). We are grateful to the support through Alexander Sartori and Alain Rico (Life Technologies).

DISCLOSURE

YGW serves/has served on scientific advisory boards for UCB Pharma and received honoraria from UCB Pharm and Desitin for participation in workshops, HL serves on scientific advisory boards for Eisai, GSK, Pfizer, UCB, and Valeant; receives industry-funded travel costs from GSK, Pfizer, and UCB; receives honoraria for speaking or educational activities from Desitin, Eisai, GSK, Pfizer, and UCB; and receives research support from Sanofi-Aventis and UCB. G.K serves on scientific advisory boards for Eisai, GSK, and UCBI and receives honoraria for speaking or educational activities from Desitin, Eisai, GSK, and UCB. TD receives honoraria for speaking or educational activities from UCB, GSK, Janssen-Cilag, Eisai, Desitin, and Novartis. The remaining authors have no potential conflicts 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.

REFERENCES


