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Gene mutational analysis in a cohort of Chinese children with unexplained epilepsy: Identification of a new *KCND3* phenotype and novel genes causing Dravet syndrome

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ABSTRACT

Purpose: This study aimed to investigate the genetic etiology of epilepsy in a cohort of Chinese children.
Methods: Targeted next-generation sequencing (NGS) was performed for 120 patients with unexplained epilepsy, including 71 patients with early-onset epileptic encephalopathies, and 16 patients with Dravet syndrome (including three patients with a Dravet-like phenotype) but without *SCN1A* pathogenic variants.
Results: Pathogenic variants of 14 genes were discovered in 22 patients (18%). A *de novo KCND3* pathogenic variant (c.1174G > A, p.Val392Ile) was identified in a boy with refractory epilepsy, psychomotor regression, attention deficit, and visual decline. Pathogenic variants in other coding genes were excluded via whole exome sequencing. This *KCND3* variant was previously confirmed to be pathogenic by Giudicessi, et al. However, the clinical profile was different: sudden death at 20 years old without any medical history of neurological disorders, nor with any diseases typically caused by *KCND3* pathogenic variants such as Brugada syndrome, spinocerebellar ataxia type 19/22 or ataxia accompanied by epilepsy. This indicates that we have identified a new *KCND3* phenotype. In addition, we also uncovered a *GRIN1* pathogenic variant and a novel *HCN1* pathogenic variant in the Dravet cohort.
Conclusion: Our study highlights the significant utility of NGS panels in the genetic diagnosis of pediatric epilepsy. Our findings indicate that *KCND3* pathogenic variants may be responsible for a wider phenotypic spectrum than previously thought, by including childhood epileptic encephalopathy. Furthermore, this study provides evidence that *GRIN1* and *HCN1* are candidate genes for Dravet and Dravet-like phenotypes.

1. Introduction

Epilepsy is one of the most common pediatric neurologic disorders, with an incidence of 7/10,000 per year before the age of two [1]. The etiology of epilepsy is diverse and complex, and genetic factors play an important role. Many genes have been associated with epilepsy, such as ion channel genes, genes related to transmitter trafficking, and genes associated with cell connections [2]. Clarifying the genetic background of epilepsy is very important for its diagnosis, treatment, and prognosis. Importantly, it also facilitates genetic counseling and prenatal diagnosis.

This clinical and genetic heterogeneity makes it difficult to uncover candidate genes with conventional Sanger sequencing. However, the increasing availability of next-generation sequencing (NGS) technologies has made it feasible to simultaneously screen a large panel of genes. In this study, targeted NGS was performed for a cohort of 120 children with unexplained epilepsy.

2. Material and methods

2.1. Patients

A cohort of 120 Chinese patients with unexplained epilepsy was recruited from the child neurology outpatient clinic of Peking University First Hospital from August 2015 to November 2016. Clinical information was collected, and genomic DNA was extracted from the

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peripheral leukocytes. Written informed consent was obtained from all patients' parents or guardians. This study was approved by the Clinical Medical Ethics Committee, Peking University First Hospital.

Approximately 70–75% of patients who fulfilled the inclusion criteria were enrolled in this study, while the remaining 25–30% of patients were uncooperative. These subjects comprised three groups. First, 71 patients were diagnosed with early onset epileptic encephalopathies (EOEEs) and fulfilled the following criteria: 1) had seizure onset within 6 months after birth, 2) their seizures were frequent and intractable and could not be eliminated by treatment with two or more anti-epileptic drugs (AEDs), 3) had developmental delay and/or intellectual disability. These 71 patients consisted of 26 patients with West syndrome, eight with the Hanefeld variant of Rett syndrome (Han-RTT), three patients with Ohtahara syndrome (OS), two patients with malignant migrating partial seizures of infancy (MMPSI), one patient with progressive myoclonus epilepsy (PME), and 31 patients with non-specific EOEEs. Second, 13 patients who met the diagnostic criteria of Dravet syndrome according to the ILAE classification of 2017 [3] were recruited, as well as three patients who did not fulfill the diagnostic criteria of Dravet syndrome but shared several Dravet-like features such as fever sensitivity; these 16 patients were defined as the Dravet cohort. *SCN1A* screening by Sanger sequencing and multiplex ligation-dependent probe amplification (MLPA) was negative in all 16 patients. Third, another 33 patients were enrolled who did not belong to the EOEE group as they had a later age of onset ranging from 7 months to 9 years old. These patients met two or more of the following criteria: 1) had two or more seizure types, 2) their seizures were frequent and intractable and could not be controlled by treatment with two or more appropriately chosen AEDs, 3) had developmental delay and/or intellectual disability with or without regression. Of these 33 patients, two patients were diagnosed with Lennox-Gastaut syndrome (LGS), two patients with Doose syndrome, one patient with PME, and 28 patients with non-syndromic epilepsy.

Metabolic disorders were ruled out by metabolic screening that included analysis of plasma amino acids and the acylcarnitine spectrum. Central nervous system infection, perinatal brain injury, and other known etiologies were excluded in all subjects. In our cohort, 89 patients underwent magnetic resonance imaging (MRI), which showed normal for 62 of the patients. The remaining 27 patients showed various minor anomalies on their MRI that were non-specific, including hypomyelination, enlarged ventricles, enlarged subarachnoid spaces, abnormalities of the cerebellar dentate gyrus, widened posterior horns of the ventricles, and global brain atrophy after adrenocorticotropic hormone (ACTH) treatment. Every patient's MRI results were assessed by more than two neurologists, and these brain anomalies were not regarded to be the cause of their epilepsy.

2.2. Targeted NGS

Library preparation including end repair, adapter ligation, and PCR enrichment was performed with the NEBNext Fast DNA Fragmentation and Library Prep Set for Ion Torrent, in accordance with the manufacturer's instructions (New England Biolabs). Targeted regions containing 437 genes in total (Supplemental material 1, Table S1) were captured with a custom SeqCap panel (Roche). The enriched libraries were sequenced on an Ion Torrent Proton sequencer (Thermo Fisher Scientific) using 200-bp single-end reads. Signal processing and base calling were carried out with Torrent Suite software v5.04 (Thermo Fisher Scientific). Reads were aligned to the human reference genome build, UCSC hg19, using TMAP. Variants were called with the Variant Caller plugin in Torrent Suite.

Variants were annotated with ANNOVAR (http://annovar.openbioinformatics.org/en/latest/). Common sites with a population allele frequency above 5% according to any of the dbSNP 138, 1000 Genomes Project, ESP6500 or ExAC databases were excluded. Given the population ethnic structure of the above databases, an in-house database containing only a Chinese population was also referenced. Variant pathogenicity was interpreted according to the ACMG Standards and Guidelines of 2015 [4]. Sanger sequencing of genomic DNA from the parents was used to determine the heredity of the variants.

2.3. Whole exome sequencing

Library preparation including end repair, adapter ligation, and PCR enrichment was carried out according to the recommendations of MyGenostics (Beijing, China). Whole-exome regions were captured with the GenCap exome capture kit (MyGenostics). The enriched libraries were sequenced on an Illumina NextSeq 500 system (Illumina) using 150-bp paired-end reads. The following bioinformatic analysis pipeline was then applied to the data: Raw reads were aligned to UCSC hg19 with BWA software. Aligned reads were processed with SAMtools and Picard following the best practice guidelines of the Genome Analysis Toolkit (GATK). Single-nucleotide variants (SNVs) and small insertion-deletions (indels) were detected with the GATK Haplotype Caller.

The variants were filtered to detect those with a population frequency of less than 5%. These SNP/indel variants were annotated with information regarding their minor allele frequency (MAF), HGMD accession number, associated diseases, and inheritance pattern. First, we particularly focused on variants in genes associated with neurologic disorders. Variants with very low MAF (< 0.002) were also regarded as candidates. Second, heterozygous variants of genes associated with dominant disorders, and homozygous/compound heterozygous variants of genes associated with recessive disorders, were marked. Variant pathogenicity was interpreted according to the ACMG Standards and Guidelines of 2015 [4]. Third, Sanger sequencing of genomic DNA from the parents was used to detect the heredity of the variants.

3. Results

Pathogenic variants of 14 genes (*KCND3*, *GRIN1*, *HCN1*, *PCDH19*, *CDKL5*, *SCN8A*, *KCNQ2*, *SCN2A*, *STXBP1*, *TBC1D24*, *CNKSR2*, *SMC1A*, *CHD2*, *KCTD7*) were detected in 22 patients, including 10 novel pathogenic variants and 14 previously reported pathogenic variants (compound heterozygous variants of *KCTD7* and *TBC1D24* were each detected in a single patient, respectively). The MAF and *in silico* pathogenicity prediction scores of the 10 novel variants are listed in Supplemental material 1 (Table S2).

In total, 18% (22/120) of patients were identified with pathogenic gene variants. The hit rates were 21% (15/71), 25% (4/16), and 9% (3/33) in the EOEE, Dravet, and non-EOEE groups, respectively. Patients with a *CDKL5* pathogenic variant accounted for the largest proportion of the EOEE cohort (8%, 6/71). The unusually high hit rate for this variant may be explained by our hospital being the largest research center for Rett syndrome in China. Patients with Rett-like features frequently attend our hospital, and patients with *CDKL5* pathogenic variants usually present some Rett features such as repetitive hand acts and other autistic behaviors [5].

The previously reported causative genes were classified into five types: ion channel/synapse, protein kinase/kinase modulator, transcription regulator, cell adhesion, and G-protein modulator (Supplemental material 1, Table S3). Ion channel genes or genes related to neurotransmitter trafficking accounted for the majority (57%, 8/14).

Notably, a *de novo* pathogenic variant (c.1174G > A, p.Val392Ile) of the *KCND3* gene was identified in our cohort. The patient (P225) was a 5-year-old boy. His seizure onset age was 1.5 years old, and his seizures were recorded as, "limbs stiffen and buckling suddenly, dribbling, cyanosis, and lost consciousness, lasting 2–3 min." His seizures occurred once or twice a month and were usually triggered by a fever. Oxcarbazepine (OXC) and valproate (VPA) were successively used without any improvement. Then, a different seizure semiology was

observed, “eyes rolling, accompanied with unilateral or bilateral limb clonus.” Levetiracetam (LEV) was applied at 3 years and 3 months, at which time the seizure frequency had attenuated to once a month. Several EEGs at different times showed abundant multifocal spikes, spike-and-wave discharges, sharp waves, and sharp-and-slow waves, primarily in the right temporal lobe. Visual decline was noticed at 3 years and 11 months of age. He was unable to position items precisely and he picked up smaller objects only by groping. Before seizure onset, his developmental milestones were normal. However, his cognition and memory ability declined following disease onset, and attention deficit was obvious. His MRI was normal at 4 years old. There was no family history of epilepsy or other neurologic diseases.

The same variant (c.1174G > A, p.Val392Ile) of *KCND3* was previously described in a patient with sudden unexplained death (SUD) at 20 years old [6]. This patient had no medical history of neurologic disorders, which was distinct from our patient. The pathogenicity of this variant was confirmed by in vitro functional analysis. To determine the cause of the difference between our patient and the patient previously reported, whole exome sequencing was performed. Heterozygous variants in 45 genes were identified, including *KCND3*. Among these variants, a further three variants predicted to be pathogenic were found in our patient, as described further in Supplemental material 1 (Table S4).

In the Dravet cohort, four patients (25%, 4/16) were discovered with pathogenic variants. An *HCN1* pathogenic variant (c.1138A > T, p.Ile380Phe) and a *PCDH19* pathogenic variant (c.932T > C, p.Val311Ala) were each identified in a patient with Dravet syndrome, respectively. In addition, in two patients (P151 and P226) who did not fulfill the diagnostic criteria of Dravet syndrome but did present some phenotypic overlap, a *GRIN1* pathogenic variant (c.2530C > T, p.Arg844Cys) and a *CHD2* pathogenic variant (c.4340delT, p.Thr1448Glnfs*29) were identified, respectively.

The clinical features of the 22 patients with pathogenic variants are summarized in Table 1 and Supplemental material 2. The reference genes used in this study are listed in Supplemental material 1 (Table S5).

4. Discussion

Our study illustrated the important role of NGS in the genetic diagnosis of pediatric epilepsy. Notably, a *de novo* missense pathogenic variant (c.1174G > A, p.Val392Ile) of the *KCND3* gene was identified in a boy with drug-resistant epilepsy, psychomotor regression, impaired vision, and attention deficit. *KCND3* encodes the voltage-gated potassium channel Kv4.3. It is widely expressed in the heart, smooth muscle, and brain [7]. The *KCND3* mutation has been linked to spinocerebellar ataxia type 19/22 (SCA19/22) and Brugada syndrome type 9 [8,9]. Recently, a different *KCND3* pathogenic variant (c.877_885dup CGCGTCTTC, p.Arg293_Phe295dup) was reported in a patient with early-onset cerebellar ataxia, intellectual disability, and epilepsy [10]. The pathogenic variant found in our patient (P225; c.1174G > A, p.Val392Ile) has previously been reported in a patient with SUD during sleep at the age of 20 years [6]. However, that patient merely had a history of syncopal episodes, with no history of arrhythmia, epilepsy or ataxia. Results from his autopsy were negative. *in vitro* functional analysis revealed that this pathogenic variant significantly increased the current density and slowed the channel inactivation [6]. However, our patient’s clinical profile was distinctly different. He presented with epilepsy, psychomotor regression, impaired vision, and attention deficit, with no medical history of cardiac attack or ataxia. Electrocardiogram at 5 years old was normal, as was his MRI at 4 years old. Whole exome sequencing ruled out other causative gene variants. This is the first report of a patient with the *KCND3* pathogenic variant presenting with epilepsy, psychomotor regression, and vision impairment but no ataxia or cardiac events. Our study, therefore, suggests that this *KCND3* pathogenic variant may contribute to a wider phenotypic spectrum than currently thought, by including childhood epileptic encephalopathy. However, this requires further investigation as whole exome sequencing has its limitations. Other genetic anomalies including copy number variants, trinucleotide repeats, and variants in non-coding regions might have been missed. Furthermore, the polygenic mode of inheritance may also be at play.

We also identified 4/16 patients in the Dravet cohort with pathogenic variants in *HCN1*, *PCDH19*, *GRIN1*, and *CHD2*. The *GRIN1* gene, which maps to 9q34.3 and encodes the glutamate ionotropic receptor NMDA type subunit 1, was recently associated with intellectual disability and epileptic encephalopathy [11–14]. Patients with *GRIN1* pathogenic variants are documented with various epileptic phenotypes, including infantile spasms, tonic and atonic seizures, generalized seizures, status epilepticus, and febrile seizures [11]. In our study, patient P151 was identified with a *de novo GRINI* pathogenic variant (c.2530C > T, p.Arg844Cys), which was previously reported to be pathogenic [11]. However, there is no detailed clinical description available regarding patients with this variant. In our patient, epilepsy was drug-resistant and fever-sensitive. Status epilepticus was also common, and the patient presented with profound developmental delay.

The *HCN1* gene is located at 5p12 and encodes the hyperpolarization-activated cyclic nucleotide-gated potassium channel 1, which contributes to spontaneous rhythmic activity in both the heart and brain [15]. Nava et al. identified this *HCN1* pathogenic variant in six unrelated patients, all of whom had drug-resistant febrile and afebrile seizures [16]. All patients had an intellectual disability of varying degrees, four had autistic features, and two had ataxia. A novel *de novo HCN1* pathogenic variant (c.1138A > T, p.Ile380Phe) was identified in our Dravet cohort. Consistent with Nava’s report, our patient (P231) presented with intractable and fever-sensitive epileptic encephalopathy. In contrast to previous reports, however, status epilepticus occurred frequently for our patient. Clinicians should pay attention to this, as status epilepticus is life-threatening and harmful to brain function. Because our patient was relatively young, no symptoms and signs of ataxia were obvious, implying that long-term tracking is necessary. To the best of our knowledge, this is only the second report of an *HCN1* pathogenic variant associated with epilepsy, and our results provide supporting evidence that this gene contributes to the Dravet spectrum.

A heterozygous pathogenic variant in *PCDH19* was also discovered in our study. This is another gene commonly linked with Dravet syndrome, with females mostly being affected [17]. Furthermore, a *CHD2* pathogenic variant was identified in a patient with a Dravet-like profile. *CHD2* has been associated with different types of childhood myoclonic epilepsy, and seizures in most patients are fever-sensitive or photosensitive [18–20]. *GRIN1*, *HCN1*, *PCDH19*, and *CHD2* should, therefore, be taken into consideration when diagnosing and treating patients with Dravet or Dravet-like phenotypes, especially those negative for *SCN1A* screening.

On the other hand, our analysis provided a more precise genetic diagnosis for several patients. For instance, a boy (P102) presented with severe psychomotor retardation and intellectual disability. Epilepsy started at 3.5 years old. Non-syndromic epileptic encephalopathy was initially diagnosed because of his “non-specific phenotype”. Through the use of our NGS panel, a nonsense pathogenic variant (c.484C > T, p.Gln162*) in *CNKSR2* was identified. *CNKSR2* (connector enhancer of KSR2) maps to Xq22.12 and is a synaptic protein. It plays an important role in neuronal proliferation, migration, and differentiation. Loss of function of CNKSR2 is associated with the epilepsy-aphasia spectrum (EAS), which is characterized by epilepsy, development delay, intellectual disability, attention deficit hyperactivity disorder (ADHD), affected speech, and continuous spike-and-slow-waves (CSWS) on EEG [21–23]. The onset age ranges from 8 days to 3.5 years. Seizures vary and can include absence seizures, febrile convulsions, and generalized tonic seizures. A retrospective study was performed for patient P102.

Table 1
Summary of clinical information of patients and mutations.

Genes	ID	age(m)	Sex	Mutation	Inheritance	*R/N*	Onset age	Seizures	Cognition/ development	Treatment	seizure-free	Diagnose
KCND3	225	60	M	c.1174G > A, p.Val392Ile	de novo	*R*	18m	Generalized seizures, focal seizures	Regression	OXC, VPA, LTG	Partially effective	Non-syn EE
GRIN1	151	29	F	c.2530C > T, p.Arg844Cys	de novo	*R*	12m	Focal seizure, SE	Delay	VPA, Ilepcimide	No	DS-like
HCN1	231	7	F	c.1138A > T, p.Ile380Phe	de novo	*N*	50d	Generalized (spasms, myoclonus), febrile seizrues, SE	Delay	LEV, VPA, PB, CZP, OXC	No	DS
PCDH19	175	22	F	c.932T > C, p.Val311Ala	father	*N*	6m	Focal seizures	Delay	PB, LEV, VPA	Yes (3 mo)	DS
CHD2	226	84	M	c.4340delT,p.Thr1448Gln fs*29	de novo	*N*	63m	Febrile seizurs, focal myoclonus	Moderate delay	VPA, LTG	Partially effective	DS-like
CNKSR2	102	57	M	c.484C > T, p.Gln162*	de novo	*N*	42m	Focal (myoclonus, atonic), SE	Moderate Delay	LEV, VPA, LTG	No	EAS
TBC1D24	215	7	M	c.1517_1519delTCA, p.Leu506_Ile507delinsLeu	father	*N*	45d	Focal myoclonus, SE	Delay	LEV, TPM, PB, VPA, clobazam, KD	No	PME
				c.116C > T, p.Ala39Val	mother	*R*						
KCTD7	230	111	M	c.440T > C, p.Leu147Pro	mother	*N*	21m	Focal myoclonus	Regression	VPA, LEV, methylprednisolone	Partially effective	PME
				c.520G > A, p.Ala174Thr	father	*N*						
CDKL5	71	11	F	c.58G > C, p.Gly20Arg	de novo	*R*	2m10d	Generalized spasm, focal seizures	Delay	TPM, VPA, VGB	No	Han-RTT
CDKL5	131	26	F	c.533G > C, p.Arg178Pro	de novo	*R*	2m	Generalized spasm	Delay	PB, OXC, LEV, CZP, ACTH	No	Han-RTT
CDKL5	161	7	F	c.533G > T, p.Arg178Leu	de novo	*R*	40d	Generalized spasm	Delay	LEV, VPA	No	Han-RTT
CDKL5	174	14	F	c.1152C > G, p.Thr384*	de novo	*R*	1m	Generalized spasm, focal seizures	Delay	VPA, LEV, TPM, CZP	No	Han-RTT
CDKL5	228	31	M	c.819delA, p.Leu273*	de novo	*N*	41d	Focal seizures, generalized spasm, clonic-tonic	delay	TPM, LTG, VPA	Partially effective	Han-RTT
CDKL5	227	21	F	c.400C > T, p.Arg134*	de novo	*R*	17d	Focal seizures, generalized spasm	delay	VPA, B6	No	Han-RTT
SCN2A	214	39	M	c.781G > A, p.Val261Met	de novo	*R*	3d	Focal seizures	Mild delay in gross motor	PB, CBZ, VPA, OXC, LTG, prednisone	No	EOEE
SCN2A	229	5	M	c.4712T > C, p.Ile1571Thr	de novo	*N*	2d	Focal seizures, generalized spasm	Delay	PB, TPM, LEV, VPA, CZP, VGB	No	OS
KCNQ2	189	5	F	c.926C > T, p.Ala309Val	de novo	*R*	2d	Focal seizures, generalized spasm	Delay	VPA, TPM, LTG	No	OS
KCNQ2	9	11	M	c.821C > T, p.Thr274Met	de novo	*R*	24h	Focal seizures, generalized spasm	Severe delay	TPM, VPA, VGB, Clobazam, CZP	No	West
SCN8A	210	8	M	c.5630A > G, p.Asn1877Ser	de novo	*R*	5m	Focal to bilateral tonic-clonic	mild delay	LEV, VPA, OXC	Yes (18 mo)	EOEE
SCN8A	134	24	F	c.4423G > A, p.Gly1475Arg	de novo	*R*	4m	Focal seizures	Delay	VPA, TPM, CZP	No	EOEE
STXBP1	70	3	F	c.388_389delCT, p.Leu130Aspfs*11	mosaic father	*R*	20d	Generalized spasm	Delay	LEV, TPM, VPA,	No	West
SMC1A	176	5	F	c.2430_2445delTGAGAATCAGAAGACT, p.Glu811Alafs*13	unknown	*N*	2.5m	Focal seizures	Delay	LEV, TPM, OXC	No	Cdl

F, female; M, male; y, years; m, months; d, days; h, hours; SE, status epilepticus; DS, Dravet syndrome; PME, progressive myoclonus epilepsy; Han-RTT, Hanefeld variant of Rett syndrome; EOEE, early-onset epileptic encephalopathy; Non-syn EE, non-syndromic epileptic encephalopathy; EAS, epilepsy-aphasia spectrum; EEOC, epileptic encephalopathy childhood-onset; Cdl, Cornelia de Lange syndrome; KD, ketogenic diet; VPA, valproate; CZP, clonazepam; CZB,carbamazepine; TPM, topiramate; LTG, lamotrigine; PB, phenobarbital; ACTH, adrenocorticotropic hormone; VGB, vigabatrin NA, not available; N, novel; R, reported.

We found that he had the same clinical course as the patients previously reported with this *CNKSR2* mutation. Apart from refractory epilepsy and psychomotor retardation, his speech delay and ADHD were also significant, although we did not initially consider this a key finding. Thus, the patient was re-diagnosed with EAS. To date, only six large intragenic deletions or complete deletions of the *CNKSR2* gene and two pathogenic point mutations of *CNKSR2* have been reported [21–24]. All of these reported variants were inherited from the healthy or mildly phenotypic mother through X-linked recessive inheritance. To the best of our knowledge, the nonsense *CNKSR2* pathogenic variant (c.484C > T, p.Gln162*) identified in our patient is the first report of a *de novo* instance of this variant, which expands the genetic spectrum of *CNKSR2* and highlights the contribution of pathogenic *CNKSR2* point mutations to the EAS spectrum.

5. Conclusion

Our study highlights the significant utility of NGS panels in the diagnosis of epileptic patients with unknown etiology, especially patients with EOEEs. Most importantly, our study revealed that refractory epilepsy, psychomotor regression accompanied by attention deficit, and visual decline is a new phenotype associated with pathogenic *KCND3* variants. We also provide evidence that *HCN1* is a candidate gene for Dravet syndrome. Additionally, we provided a more precise diagnosis for several patients that would not otherwise have been achieved simply by clinical assessment and conventional diagnostic approaches.

Conflict of interest

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

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.seizure.2019.01.025.

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