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Diagnostic yield of targeted massively parallel sequencing in children with epileptic encephalopathy

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ABSTRACT

Purpose: To report our institutional experience of targeted massively parallel sequencing (MPS) testing in children with epilepsy.
Method: We retrospectively analysed the yield of targeted epileptic encephalopathy (EE) panel of 71 known EE genes in patients with epilepsy of unknown cause, who underwent clinical triage by a group of neurologists prior to the testing. We compared cost of the EE panel approach compared to traditional evaluation in patients with identified pathogenic variants.
Results: The yield of pathogenic variants was 28.5% (n = 30/105), highest in early onset EE <3 months including Ohtahara syndrome (52%, n = 10/19) and lowest in generalized epilepsy (0/17). Patients identified with pathogenic variants had earlier onset of seizures (median 3.6 m vs 1.1y, $p < 0.001$, OR 0.6/year, $P < 0.02$) compared to those without pathogenic variants. Pathogenic/likely pathogenic variants were found in *ALDH7A1* (2), *CACNA1A* (1), *CDKL5* (3), *FOXG1* (2), *GABRB3* (1), *GRIN2A* (1), *KCNQ2* (4), *KCNQ3* (1), *PRRT2 (1)*, *SCN1A* (6), *SCN2A* (2), *SCN8A* (2), *SYNGAP1* (1), *UBE3A* (2) and *WWOX* (1) genes. This study expands the inheritance pattern caused by *KCNQ3* mutations to include an autosomal recessive severe phenotype with neonatal seizures and severe developmental delay. The average cost of etiological evaluation was less with early use of EE panel compared to the traditional investigation approach ($5990 Australian dollars (AUD) vs $13069 AUD ; p = 0.02) among the patients with identified pathogenic variants.
Conclusion: Targeted MPS testing is a comprehensive and economical investigation that enables early genetic diagnosis in children with EE. Careful clinical triage and selection of patients with young onset EE may maximize the yield of EE panel testing.

1. Introduction

Epileptic encephalopathies are a large group of disorders of refractory epilepsy usually with onset in infancy or early childhood associated with abnormal EEG, severe cognitive and behavioural impairment, above that expected from the underlying pathology alone. The age-adjusted incidence of epilepsy ranges from 24 to 53 per 100,000 person-years [1]. Recent small population based studies show that one third of children presenting with epilepsy before 36 months are medically intractable and 36% of epilepsy commencing before 24 months manifest as epileptic encephalopathy [2,3]. Evaluation of poorly controlled epilepsies in infants and children often remains a challenge in clinical practice and involves performing a number of metabolic and imaging investigations to identify the cause [4–6].

Abbreviations: EE, epileptic encephalopathy; EEG, electro encephalogram; MRI, magnetic resonance imaging; MPS, massively parallel sequencing; VOUS, variant of unknown significance; EOEE, early onset epileptic encephalopathy; GGE, genetic generalised epilepsy.

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Although some genetic encephalopathies have distinctive electroclinical features and comorbidities, a substantial number of patients with epileptic encephalopathy do not have phenotypes that fit into specific epilepsy syndromes, and this hampers a clear clinical diagnosis and prevents straightforward genetic testing [7]. Unlike genetic generalized epilepsies, epileptic encephalopathies can be monogenic and identification of the underlying causative gene helps with diagnosis [8].

With the advent of sophisticated molecular diagnostic techniques, rapid growth in gene discovery for epileptic encephalopathies has occurred and broadened gene-specific phenotypes. We review our institutional experience of MPS using the EE panel and then highlight the importance of clinical triage and phenotyping. Clinical benefits of EE panel testing are illustrated and a diagnostic algorithm including EE panel testing for investigation of children with epileptic encephalopathies is proposed.

2. Methods

The study was approved by Sydney Children's Hospital Network (SCHN) ethics committee no LNR/16/SCHN/254.

2.1. Patients

The targeted MPS EE panel was introduced in late 2013 as a clinical diagnostic test at our centre. We retrospectively analysed the yield of the EE panel in patients with epilepsy who underwent the EE panel using MPS testing between January 2014 and September 2016 at The Children's Hospital at Westmead (CHW). All the patients underwent detailed clinical triage by a group of neurologists at monthly meetings prior to the testing, in order to ensure appropriate referrals. The patients were prioritized for EE panel testing if they had ongoing seizures, a persistently abnormal EEG and no cause was found despite investigations or if a specific monogenic epilepsy was suspected. These patients underwent MRI of the brain and a range of metabolic and genetic testing prior to proceeding to EE panel testing as part of routine clinical evaluation. Children with cortical malformations of the brain and those with pathogenic copy number variants on CGH microarray were excluded in this study. The clinical details regarding electroclinical syndrome, type of seizures, age of onset, abnormal neurological examination, developmental delay prior to and after onset of epilepsy, EEG, and MRI results were reviewed. Epilepsy syndromes were classified according to the Organization of the International League Against Epilepsy Commission on Classification and previously used classifications in epileptic encephalopathy studies [7,9].

2.2. Massively parallel sequencing assay and variant interpretation

Using the Illumina TruSight One panel, a diagnostic panel of 47 EE genes with clear evidence for causing epilepsy was designed in conjunction with clinical, molecular geneticists and neurologists, which was later expanded to 71 genes due to the discovery of new genes associated with EE *(AARS, ADSL, ALDH7A1, ALG13, ARHGEF9, ARX, ASNS, BRAT1, CACNA1A, CACNB4, CASK, CDKL5, CHD2, CNTNAP2, DCX, DNM1, FASN, FLNA, FOXG1, GABRA1, GABRB3, GABRG2, GAMT, GPHN, GRIN1, GRIN2A, GRIN2B, HCN1, HDAC4, HNRNPU, IQSEC2, KCNQ2, KCNQ3, MAGI2, MBD5, MECP2, MEF2C, MOCS1, MOCS2, MTHFR, NEDD4L, NRXN1, NTNG1, PCDH19, PIGO, PLCB1, PNKP, PNPO, POLG, PRICKLE1, PRRT2, RYR3, SCN1A, SCN1B, SCN2A, SCN8A, SCN9A, SLC25A22, SLC2A1, SLC9A6, SPTAN1, ST3GAL3, STXBP1, SUOX, SYN1, SYNGAP1, TBC1D24, TCF4, UBE3A, WWOX, ZEB2)*.

Target enrichment for samples was performed using the Illumina TruSight One sequencing panel (Illumina Inc., 2013–2016) containing probes to capture the exonic regions of 4813 genes associated with a clinical phenotype. The samples were sequenced on an Illumina HiSeq 2500 or Illumina NextSeq 500 (Illumina Inc.) with 2×150 bp paired-end reads (Illumina Inc.). Alignments and variant calls were generated using NextGene software (v2.4.1, 2015) and variant calls (with coverage <15X) were limited to the genes of interest. Variants were interpreted using Alamut-Batch (Version 1.4.0, 2015). Variants were annotated for minor allele frequencies in the Exome Aggregation Consortium (ExAC) database (Version 0.3), and heterozygous variants with minor allele frequencies >0.01 (1%) were filtered out. Variants were classified as pathogenic/likely pathogenic/VOUS/likely benign/benign according to the 2015 American College of Medical Genetics and Genomics (ACMG) guidelines [10] based on a combination of previous reports in the literature, computational analysis, functional, and population data. Nonsense, frameshift, and canonical splice-site variants were considered strongly indicative of pathogenicity in a gene where loss of function is a known mechanism of disease. For missense variants, Alamut Visual (Version 2.7) was used for individual variant analyses, providing computational algorithms for SIFT, PolyPhen-2 (Version 2.2.2, 2012) and MutationTaster (Version 2, 2012). Variants that were classified as pathogenic or likely pathogenic were validated using Sanger sequencing in the proband. Segregation studies were performed in family members when available. All the cases were discussed with molecular genetic scientists in a meeting following EE panel results to compare genetic testing results with the individual clinical phenotype. The complex cases were discussed in a multidisciplinary team including clinical geneticists, molecular genetic scientists, genetic counsellors and other clinicians.

2.3. Clinical benefits and cost analysis

We analysed the period and the cost of diagnostic evaluation amongst patients with pathogenic variants who were evaluated with the traditional approach prior to the introduction of the EE panel (pre MPS availability, n = 9) with those who were investigated after the introduction of EE panel with early EE panel testing (post MPS availability, n = 9) using information provided by the clinical costing centre in Management Support Analysis Unit at our institute. The analysis was limited to the patients where genetic diagnosis could not be predicted based on clinical presentation. We excluded 5 patients in pre MPS group and 7 patients in Post MPS group in whom diagnosis was already suspected by the treating clinician based on electroclinical phenotype and biochemical testing. The etiological investigations were performed either as inpatient admission/outpatient follow up and were grouped into 4 categories as discussed below: 1) metabolic tests including CSF studies (first line and second line), 2) neuroimaging, 3) admissions and procedures, 4) presurgical evaluation including video telemetry monitoring for surgical indications, PET and SPECT and 5) other genetic testing.

2.4. Statistical analysis

Statistical analysis was performed and graphs were composed using Graph Pad Prism software version 6. Categorical variables were compared using Fisher's exact test and continuous variables were compared using Mann Whitney test and two sample T test depending on the normality of data distribution. Clinical parameters (age of onset of epilepsy and electroclinical syndrome) were analysed using univariate regression model to predict the presence of pathogenic variants among patients who underwent EE panel testing.

3. Results

3.1. EE panel genetic details

One hundred and sixty nine patients were discussed in the neurology clinical triage meeting. Twenty eight patients were not

tested either due to enrolment in other research genetic studies, or because the diagnostic yield was considered low in the clinical triage meeting by the neurologists. Of the 141 patients who underwent EE panel testing, results were available for 105 patients at the time of this report. Thirty out of 105 patients were identified with pathogenic variants and likely pathogenic variants in 15 genes that could explain the underlying cause of epilepsy. Table 1 summarizes the results of EE panel analysis in the 30 patients (28.5%) identified with a causative gene. Thirty two percent of patients had VOUS's (n = 34/105) and 39% patients did not have any pathogenic variants or VOUS (n = 41/105). Patients identified with a pathogenic variant were younger at testing (median age, 2.3y vs 4.98y, p = 0.03) and had earlier onset of seizures (median 3.6 m vs 1.1y, $p < 0.001$; Odds ratio 0.6 per year, confidence interval 0.4–0.9, p = 0.02) compared to those with no pathogenic variants and VOUS.

Among 105 patients, 37 (35.2%) had the 47 gene panel whilst 68 (64.8%) patients had the 71 gene panel. Updating the panel with newly discovered EE genes resulted in a genetic diagnosis in only 4 patients *(CACNA1A, KCNQ3, GABRB3 and WWOX)*. The number of patients identified with a pathogenic variant in each gene is shown in Fig. 1a. The most common pathogenic variant type was missense (13/30, 43.3%). Other pathogenic variants included frameshift (8/30, 24.2%), nonsense, (4/30, 12.1%), and splice-site variants (5/30, 15.2%). In 18 patients, the variants were novel and 12 cases had previously reported pathogenic variants. In 14 cases the variants were shown to be *de novo*. Four patients showed an autosomal recessive inheritance pattern [*ALDH7A1* (2), *WWOX (1), KCNQ3 (1)*]. Case 3 showed autosomal dominant inheritance with multiple affected family members with intellectual disability and absence epilepsy and the identified *CACNA1A* variant was also present in the proband's affected sister. The MPS data identified 2 patients (case 5 and 13) that were somatic mosaics for pathogenic variants in *CDKL5* and *KCNQ2* respectively.

Fig. 1b shows the yield of EE panel testing according to individual electroclinical syndrome. Pathogenic yield was higher in benign neonatal/infantile epilepsy (BNE, n = 3/3), early onset epileptic encephalopathy (EOEE) including Ohtahara syndrome (OS) (10/19, 52%), and Dravet syndrome (n = 6/10, 60%) compared to epileptic encephalopathy not otherwise specified (EENOS) (7/27, 26%) and infantile spasms (1/8, 12%). Only one patient with myoclonic absence epilepsy had a pathogenic variant in the *SYNGAP1* gene. EE panel testing did not identify any pathogenic variants in patients with early onset, refractory absence or other generalised epilepsy (n = 17), or focal epilepsy (n = 5).

3.2. Clinical phenotyping details

Details regarding electroclinical syndrome, seizure types, EEG, neuroimaging and cognitive outcomes are presented in Supplementary Table 1. Clinical features were suggestive of the underlying genetic cause in one third of patients [Dravet syndrome, *SCN1A* (n = 6); atypical absence and paroxysmal tonic upgaze with a positive family history, *CACNA1A* (n = 1) [11]; pyridoxine dependent epilepsy, *ALDH7A1* (n = 2); Angelman syndrome, *UBE3A* (n = 1); and benign familial neonatal seizures, *KCNQ2* (n = 1)]. Five patients had movement disorder (*FOXG1* = 2, *SCN8A* = 1, *KCNQ2* = 1, and *STXBP1* = 1) in addition to epilepsy.

Diagnosis was not suspected in two thirds of the cases with pathogenic variants details of which are presented in Supplementary Table 1. In four cases, electroclinical and imaging features of the cases were retrospectively reviewed after obtaining the genetic diagnosis and found to be consistent with clinical phenotype described in the literature (*FOXG1*, Case 7 & 8; *SYNGAP1,* Case 27 and *WWOX*, Case 30*;* Supplementary Table 1). Case 9 with the *GABRB3* variant presented with refractory spasms and was unusually hypersensitive to vigabatrin in the form of decreased alertness.

Three cases had atypical presentations that expand the phenotypes of known pathogenic variants. A 10 yr old boy (Case 5) presented with Rett syndrome-like features who was diagnosed to be mosaic for a pathogenic variant in the *CDKL5* gene (c.533G > A; p.Arg178Gln). The patient with a *UBE3A* pathogenic variant (Case 28) presented with severe intellectual disability, speech delay, mild ataxia, tremors, epilepsy with non-convulsive status and a characteristic shark tooth wave appearance on EEG, but lacked characteristic physical features of Angelman syndrome.

We also describe a case of familial neonatal seizures and intellectual disability (Case 15) due to an autosomal recessive homozygous frameshift variant in *KCNQ3* (Fig. 2). This is a 4-year-old boy who was born to consanguineous parents of Lebanese ancestry following an uncomplicated delivery. He presented with neonatal clonic seizures on day 1 and received phenobarbitone for the first 2 months of life. He had a subsequent recurrence of focal seizures at 10 months and remains seizure free on levetiracetam. His neonatal EEG and EEG at 2 yrs showed multifocal spike and wave discharges with abnormal slow background. He was noted to have hypotonia, severe developmental delay and could only stand with support and remained nonverbal at the age of 4 yrs. He had extensive investigations including urine metabolic screen, CSF studies, lactate, ammonia, CGH microarray, EMG, nerve conduction studies and muscle biopsy, all of which were normal. His MRI showed nonspecific periventricular high signal in parieto-occipital white matter. His elder brother, a 12 year boy, presented with seizures on day 7 followed by recurrence of focal seizures at 12 years. He had mild intellectual disability, learning and speech difficulties, and behavioural problems. His 6 year old brother also presented with neonatal seizures on day 7 and his neonatal EEG was normal. He had mild speech delay in follow up. His 3 month old sister was unaffected. There was no history of seizures or neurological problems in his parents and extended family members. MPS testing showed a homozygous frameshift variant in *KCNQ3* (c.1220_1221delCT p.Ser407Phefs*27), which is considered to be likely pathogenic according to the ACMG guidelines for the classification of sequence variants. Familial segregation analysis confirmed homozygosity in the affected siblings and the parents were shown to be heterozygous for the same variant (Fig. 2).

3.3. Clinical benefits and cost evaluation of EE panel diagnosis in patients with identified pathogenic variants (Table 2)

Having a genetic diagnosis helped in reproductive planning (n = 5), guiding medication management (n = 7), and avoiding further presurgical evaluation and subsequent surgery in (n = 2), as shown in Table 2. The finding of a pathogenic *CDKL5* variant helped in prenatal counselling and testing in case 4 whose mother was 8 weeks pregnant. Similarly identification of *ALDH7A1* in case 1 & 2 helped counsel families about life long treatment with pyridoxine and risk of recurrence in extended family members. Identification of a pathogenic variant in *SCN1A* in young infants with Dravet syndrome (Case 18, 19 and 21 aged 2yrs, 8 m and 1.7yr) helped avoid carbamazepine and other sodium channel blockers that might exacerbate seizures and to counsel family regarding the course of the epilepsy. Case 26 with a *SCN8A* variant was extremely responsive to phenytoin and needed to be maintained on high therapeutic doses (100–140 microMol/L) to prevent recurrent intensive care unit admissions due to status epilepticus. Case 6 presented with focal seizures and spasms with predominant left temporal epileptic discharges. Based on MRI and PET, dysplasia involving left temporo-parietal and occipital junction was suspected. However her EE panel showed a pathogenic variant in *CDKL5*, which helped us avoid further presurgical evaluation and to counsel the family appropriately. Similarly Case 25 presented with

Table 1
Genotype details of pathogenic variants and likely pathogenic variants.

Case N (Sex)	Curr age/ age of onset	Epilepsy Syndrome	Gene (transcript) and Inheritance pattern*	Nucleotide change**	Predicted protein change	Family testing	Allelic frequency (EXAC)	Type of variant	Previous report
Case 1 (M)	2y/7d	EOEE	*ALDH7A1* (NM_001182.3) AR	c.1475_1476insC; c.1547A > G	p. Gly493Trpfs*19; p.Tyr516Cys	Compound heterozygosity confirmed	none 8/ 122938	Frameshift Missense	Novel
Case 2 (M)	11y/7d	EOEE	*ALDH7A1* (NM_001182.4) AR	c.834G > A; c.865A > G	p.Val278Val; p. Arg289Gly	Compound heterozygosity confirmed	7/121412 alleles none	Splicing Missense	[40]
Case 3 (M)	6y/5m	EE NOS	*CACNA1A (NM_001127222.1)* AD	c.4051C > T	p.Arg1351*	Detected in affected sister	none	Nonsense	Novel
Case 4 (F)	1.6y/2m	EOEE	*CDKL5* (NM_003159.2) XLD	c.533G > A	p.Arg178Gln	*De novo*	none	Missense	RettBASE [41]
Case 5 (M)	10y/2m	EOEE	*CDKL5* (NM_003159.2) XLD	c.533G > A	p.Arg178Gln	Not performed proband mosaic	none	Missense	RettBASE [41]
Case 6 (F)	2y/1m	EOEE	*CDKL5* (NM_003159.2) XLD	c.1156del	p. Ala386Glnfs*107	*De novo*	none	Frameshift	Novel
Case 7 (M)	3y/11m	EE NOS	*FOXG1* (NM_005249.4) AD	c.946del	p. Leu316Cysfs*10	*De novo*	none	Frameshift	Novel
Case 8 (F)	6y/1m	EOEE	*FOXG1* (NM_005249.4) AD	c.1403del	p. Ser468Leufs*20	Not found in mother, father not available	none	Frameshift	Novel
Case 9 (F)	1.8y/4m	IS	*GABRB3* (NM_000814.5) AD	c.229G > A	p.Glu77Lys	*De novo*	none	Missense	Novel
Case 10 (F)	8y/3.8y	EAS	*GRIN2A* (NM_000833.4) AD	c.1652-1G > A	p.?	*De novo*	none	Splicing	Novel
Case 11 (F)	1.6y/2d	OS	*KCNQ2* (NM_172107.2) AD	c.881C > T	p.Ala294Val	*De novo*	none	Missense	[42]
Case 12 (M)	2y/1d	OS	*KCNQ2* (NM_172107.2) AD	c.917C > T	p.Ala306Val	*De novo*	none	Missense	Novel
Case 13 (M)	6m/7d	BNE	*KCNQ2* (NM_172107.2) AD	c.1764-5G > A	p.?	*De novo*, mosaic	none	Splicing	Novel
Case 14 (F)	0.3y/3m	BIE	*KCNQ2* (NM_172107.2) AD	c.1764A > T	p.Arg588Ser	Not available	none	Missense	[43]
Case 15 (M)	4.2y/1d	EOEE and severe developmental delay	*KCNQ3* (NM_004519.3) AR	c.1220_1221del (Hom)	p. Ser407Phefs*27	Homozygosity confirmed; 2 affected siblings homozygous	none	Frameshift	novel
Case 16 (M)	0.8y/6m	BIE	*PRRT2* (NM_145239.2) AD	c.649dupC	p.Arg217Profs*8	Not available	0.6% (401/ 64034 alleles)	Frameshift	[44]
Case 17 (M)	6y/7m	Dravet	*SCN1A* (NM_001165963.1) AD	c.230T > C	p.Leu77Pro	*De novo*	none	Missense	Novel
Case 18 (M)	2y/2.5m	Dravet	*SCN1A* (NM_001165963.1) AD	c.301C > T	p.Arg101Trp	Not available	none	Missense	SCN1A database
Case 19 (M)	0.7y/4m	Dravet	*SCN1A* (NM_001165963.1) AD	c.1702C > T	p.Arg568*	Not available	none	Nonsense	SCN1A database
Case 20 (F)	3y/7m	Dravet	*SCN1A* (NM_001165963.1) AD	c.2593C > T	p.Arg865*	Not available	none	Frameshift	SCN1A database
Case 21 (M)	1.7y/4m	Dravet	*SCN1A* (NM_001165963.1) AD	c.2794T > A	p.Trp932Arg	*De novo*	none	Missense	Novel
Case 22 (F)	17.3y/4m	Dravet	*SCN1A* (NM_001165963.1) AD	c.3948G > C	p.Arg1316Ser	Not available	none	Missense	[45]
Case 23 (M)	15y/18m	EENOS	*SCN2A* (NM_021007.2) AD	c.605 + 1G > A	p.?	Not available	none	Splicing	Novel
Case 24 (F)	10y/14m	EENOS	*SCN2A* (NM_021007.2) AD	c.823C > T	p.Arg275*	*De novo*	none	Nonsense	Novel
Case 25 (F)	3y/8m	EENOS	*SCN8A* (NM_014191.3) AD	c.5597G > A	p.Arg1866Gln	*De novo*	none	Missense	Novel

Table 1 (*Continued*)

Case N (Sex)	Curr age/ age of onset	Epilepsy Syndrome	Gene (transcript) and Inheritance pattern*	Nucleotide change**	Predicted protein change	Family testing	Allelic frequency (EXAC)	Type of variant	Previous report
Case 26 (F)	10y/5m	EENOS	*SCN8A* (NM_014191.3) AD	c.5614C > T	p.Arg1872Trp	*De novo*	none	Missense	Novel
Case 27 (F)	5y/6m	MAbE	*SYNGAP1* (NM_006772.2) AD	c.439C > T	p.Gln147*	*De novo*	none	Nonsense	Novel
Case 28 (M)	9.6y/8y	Angelman	*UBE3A* (NM_130838.1) AD	c.1588_1590del	p.Lys530del	Not available	none	In frame deletion	UBE3A database
Case 29 (M)	6.2 y/2y 6m	EENOS	*UBE3A* (NM_000462.3) AD	c.1699G > A	p.Glu567Lys	*De novo*	none	Missense	Novel
Case 30 (M)	0.75y/1m	EOEE	*WWOX* (NM_016373.3) AR	c.606-1G > A (Hom)	p.?	Homozygosity confirmed	none	Splicing	[35]

Abbreviations: EOEE – Early Onset Epileptic Encephalopathy; EE NOS – Epileptic Encephalopathy Not Otherwise Specified; Dravet – Dravet Syndrome; MAbE – Myoclonic Absence Epilepsy; EAS – Epilepsy aphasia spectrum (LKS, CSWS, Atypical BPE); IS – Infantile spasm; OS – Ohtahara Syndrome; BNE – benign neonatal epilepsy; BIE – benign infantile epilepsy; M – male; F – female; d – days; m – months; y – years; AD – autosomal dominant; AR – autosomal recessive; XLD – X linked dominant; *inheritance pattern reported in OMIM; ** Hom – homozygous, all other variants heterozygous; RettBASE (http://mecp2.chw.edu.au/); SCN1A database (http://www.molgen.vib-ua.be/scn1amutations/Home/Default.cfm); UBE3A database (https://secure.ngrl.org.uk/LOVDv.2.0/home.php?select_db=UBE3A).

right focal seizures at 8 months of age and the EEG showed predominant left sided discharges. A dysplasia in the left superior frontal sulcus was suspected based on MRI and co-registration with functional studies including PET and SPECT. Her EE panel test revealed a likely pathogenic variant in *SCN8A*, which helped avoid surgery in this case.

The cost analysis was limited to the patients where genetic diagnosis could not be predicted based on the clinical presentation. Among the patients with pathogenic variants, the patients who underwent EE panel testing due to the earlier availability of MPS testing (n = 9), were younger at diagnosis (median 2.2 vs 8.6 years) and had a shorter period of evaluation (median 2.1 yr vs 6.5 years) compared to the patients who were evaluated with traditional investigations prior to the availability of EE panel (n = 9). The average cost of etiological evaluation was less with early availability of the EE panel compared to the traditional investigation approach ($5990 AUD post MPS availability vs $13069 AUD pre MPS availability; p = 0.02). Overall the cost of the etiological evaluation for epilepsy was significantly higher compared to cost of EE panel testing ($1500 AUD). The identification of a pathogenic variant(s) on the EE panel testing reduced the need for surgical evaluation including surgical video telemetry, PET and SPECT scans, repeat MRIs needing general anaesthesia and admissions and other invasive procedures such as muscle biopsy as highlighted in Supplementary Table 2.

4. Discussion

With advanced sophisticated genetic technology and reduction in costs, MPS is increasingly being used in clinical practice. Several studies on targeted EE panels of 35–265 genes have been reported in the literature with diagnostic yields ranging between 10% and 48.5% [12–17]. In a large study of 500 patients with EE, Carvill et al reported a 10% yield using a gene panel of 19 known and 46 candidate genes and the yield in Dravet syndrome and EOEE was reported to be 21% [14]. In another study of 400 patients with early-onset seizure disorders and/or severe developmental delay using a panel of 45 genes, 18% had a causative pathogenic variants identified with a diagnostic rate of 39% in those with seizure onset within the first 2 months [18]. The variability in diagnostic yield may be explained by the number of genes included in the panel, and the phenotypes of the patients selected for the analysis. At our centre, the diagnostic yield of targeted MPS (EE panel) was higher in our cohort compared to previous studies, possibly due to the clinic triage peer review process and careful selection of cases for testing. When compared to the use of targeted panels, the diagnostic yield in exome sequencing ranged from 11 to 72% and studies with a higher yield have been limited by a small number of patients [19–22]. However compared to exome sequencing, the EE panel approach offers improved coverage for clinically relevant genes, and reduces the identification of incidental findings and associated ethical issues in patient follow up.

Prioritizing children with EOEE for EE panel testing may be more economical in the clinical diagnostic setting compared to genetic generalized epilepsies (GGEs). The lower yield observed in GGE may be reflective of polygenic inheritance. Studies applying whole exome sequencing or targeted gene panels on large cohorts of patients with GGEs have failed to pinpoint the involvement of strong causative genes and contributory genetic variants [23,24].

The causative genetic diagnosis was not suspected in two thirds of the cases with pathogenic variants prior to EE panel testing, which highlights the genetic heterogeneity associated with given clinical phenotypes [7]. Our cases add to the existing literature outlining the complex genotype-phenotype relationships in the epileptic encephalopathies (Supplementary Table 1) [14,18,21,25,26]. In addition, we describe cases with atypical presentation that expand phenotypes of known pathogenic variants (*CDKL5, UBE3A and KCNQ3)* [27,28]. Case 15 had familial neonatal seizures and intellectual disability and had a homozygous frameshift variant in *KCNQ3* (Fig. 2). The variant showed an autosomal recessive inheritance pattern; all three affected siblings were shown to be homozygous for the variant and the unaffected parents were both heterozygous for the variant. *KCNQ3* encodes a sodium channel, Kv7.3, and pathogenic variants in this gene are associated with autosomal dominant benign familial neonatal seizures (BFNS) and all reported variants described to date are heterozygous missense changes [29,30]. In the absence of functional data, the effect that the *KCNQ3* variant (c.1220_1221delCT p.Ser407Phefs*27) has on the protein is unknown. A study has shown that a 25% loss of channel function is sufficient to cause BFNS [31]. It is hypothesized that this variant may lead to a partially functional protein resulting in unaffected heterozygous carriers and a loss of function in individuals with homozygous variants causing a clinical phenotype. Functional studies would be required to prove this theory. Furthermore, it is

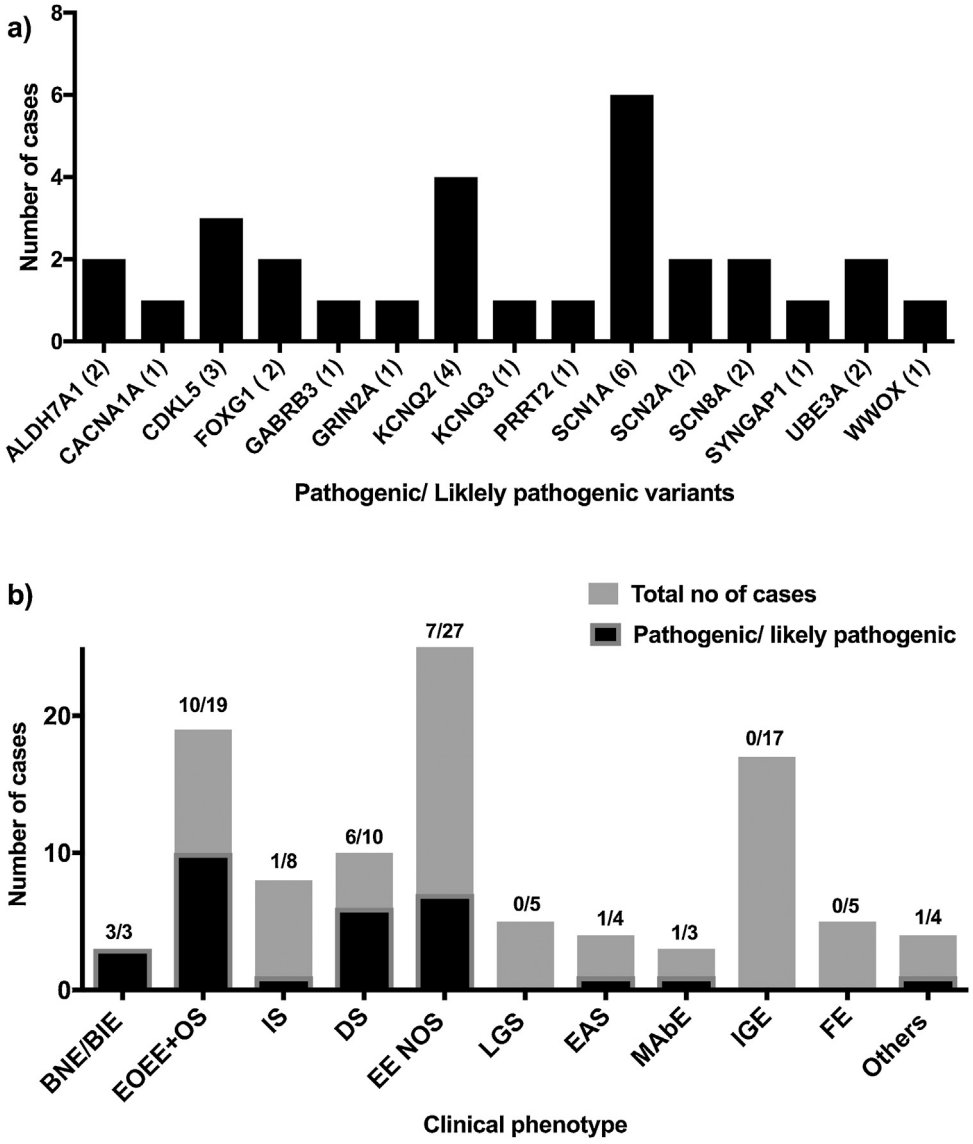

Fig 1. (a) Genes with pathogenic and likely pathogenic variants seen in children with epilepsy. (b) The yield of pathogenic and likely pathogenic variants according to electroclinical phenotype.
Abbreviations: BNE – benign neonatal epilepsy; BIE – benign infantile epilepsy; OS – Ohtahara Syndrome; EOEE – Early Onset Epileptic Encephalopathy; IS – Infantile spasm; DS – Dravet Syndrome; EENOS – Epileptic Encephalopathy Not Otherwise Specified; LGS – Lennox Gastaut syndrome; EAS – Epilepsy aphasia spectrum (LKS, CSWS, Atypical BPE); MAbE – Myoclonic Absence Epilepsy; IGE – Idiopathic generalised epilepsy; FE – focal epilepsy.

possible the parents may have been affected in the neonatal period despite the absence of an obvious family history.

In view of genotypic heterogeneity, clinical phenotyping is central to interpretation of the relevance of a genetic finding in a patient, and this will require more neurologists to develop expertise in EE panel testing and work in collaboration with molecular genetic scientists. The *GABRB3* variant in case 9 was initially reported as a VOUS. The information at the time of the report indicated that pathogenic variants in *GABRB3* were associated with a generalised epilepsy phenotype which did not correlate with the phenotype seen in case 9. A new study reported *GABRB3* pathogenic variants in children presenting with severe early onset epileptic encephalopathy and an unusual hypersensitivity to vigabatrin [32]. This prompted parental testing which showed the VOUS was *de novo* in the proband and the variant was reclassified as likely pathogenic. This case highlights the importance of revisiting VOUS with expanding knowledge about genetic epileptic encephalopathy [32]. The reason for the unusual reaction to vigabatrin in this case is not clear, and may be related to the hypersensitivity of affected receptors to GABA neurotransmitter release [33]. Reverse phenotyping of cases following genetic diagnosis helped confirm the underlying cause in 4 cases, which emphasizes the importance of careful review of clinical phenotype after the genetic diagnosis [14,34,35].

With the availability of EE panel testing, the genetic diagnosis was obtained as young as 7 months (Case 6), which helped a family to identify relevant support groups and avoid further investigations. Some of these patients (Case 6 and 25) had focal epilepsy/spasms consistent with a potential structural cause that might need presurgical evaluation and having a genetic diagnosis was useful in deferring surgery [36]. The therapeutic response to high dose phenytoin in the patient with the *SCN8A* variant (Case 26) is reported in the literature emphasizing the importance of a molecular diagnosis [37]. Our analysis suggests that the cost saving of utilizing EE panel MPS testing will be significant in patients in whom we have found a genetic cause. With a shorter turn around time for MPS, the investigations listed in Supplementary Table 3 could be avoided thereby reducing the cost of

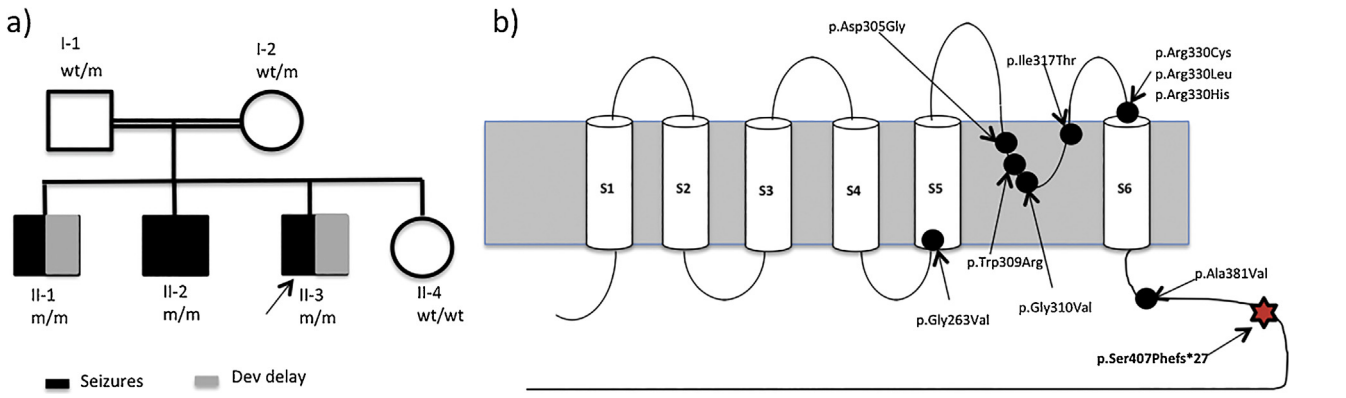

Fig. 2. a) Family pedigree of case 15 with *KCNQ3* likely pathogenic variant. The pedigree shows autosomal recessive inheritance pattern and a wide phenotypic variability. WT corresponds to the wild type allele and m denotes the presence of the *KCNQ3* variant (c.1220_1221del,(p.Ser407Phefs*27)). Index case presented with neonatal seizures and severe intellectual disability. The first sibling (II-1) presented with neonatal seizures and had mild intellectual disability whereas the second sibling (II-2) presented with benign neonatal seizures with normal EEG and mild speech delay. Third sibling (II-4) was unaffected. b) A schematic representation of Kv7.3 showing all previously reported pathogenic variants to date (black circles). All previously reported variants were heterozygous missense variants in contrast to the homozygous frame shift variant identified in the index case (red asterisk).
Abbreviations: dev, development.

Table 2
Clinical benefits and cost efficacy of EE panel testing.

Clinical Benefits	Case details
Earlier age at diagnosis	Median 2.2 yr vs 8.6 y ($p < 0.001$)
Shorter period of evaluation (Post MPS availability vs Pre MPS availability)	Median 2.1 yr vs 6.5yr ($p < 0.001$)
Reproductive planning	*CDKL5* (Case 4 and 6), *WWOX* (Case 30) and *ALDH7A1* (Case 1 and 2)].
Management implications	*SCNA1* (Case 18, 19 and 21), avoiding sodium channel blockers *SCN8A* (Case 26), higher doses of phenytoin *KCNQ2* (Case 11), continue carbamazepine *ALDH7A1* (Case 1 and 2), continue pyridoxine
Avoid further pre surgical evaluation and epilepsy surgery	*SCN8A* (Case 25) and *CDKL5* (Case 6)
Cost savings	Mean cost of investigations in traditional pathway pre MPS availability was higher ($13069 AUD; range $3325-$28,443 AUD) compared to the early use of EE panel testing in post MPS availability period ($5990 AUD ; range $2236-$8031 AUD), *$p < .02$).

*Two sample *t* test. AUD, Australian dollar

investigations in at least in a third of patients, given the diagnostic yield of MPS is 28.5%. A larger study looking at diagnostic evaluation and cost benefits of all patients enrolled for EE panel testing is planned for the future and is beyond the scope of this current study.

As a result of clinical benefits and cost benefits described in Table 2 and Supplementary Table 2, EE panel testing influenced our clinical practice, and we therefore propose a diagnostic algorithm that utilizes the targeted MPS approach for the genetic evaluation of patients with EE (Fig. 3). This begins with careful clinical phenotyping based on age of onset, seizure types, presence of developmental delay, abnormalities on neurological examination, family history and the results of selected baseline investigations such EEG, brain MRI, baseline treatable metabolic screening investigations and CGH microarray. If the above investigations do not reveal the etiology, the patients should be considered for EE panel testing using MPS, with subsequent careful consideration of whether the identified genotype explains the patient's clinical phenotype. Those with negative MPS results could move on to further testing such as whole exome or whole genome sequencing. This stepwise approach helps to reduce the cost of investigations, minimises the detection of gene variants unrelated to the primary reason for ordering the test, saves clinician and scientist's time spent in interpretation, and may simplify the need for extensive pretest genetic counseling. With rapid advances in high throughput technology, decreased cost and turn around time in future, we hope that MPS will be used along with other first line investigations in a child with difficult to control epilepsy, which saves cost and reduces time to diagnosis.

The strength of the study is that we used rigorous criteria for case selection for EE panel testing and the calling variants on the EE panel as pathogenic. EE panel testing is associated with number of limitations. One concern is that a large targeted panel will result in detection of many VOUS's, interpretation of which is difficult in the absence of information on pathogenicity in genetic registries and the lack of functional studies. Even though the EE panel is comprehensive, the panel does not cover some of the genes associated with familial focal epilepsy, idiopathic generalised epilepsy, and the EE genes that are not available on TrueSight One platform. In addition, there is also the need for regular updating of the panel as new genes are discovered. Despite this, as approximately 4800 pathogenic genes are already sequenced as part of the TruSight One panel, it is possible to reevaluate the data for additional genes for patients who were initially panel negative, although novel genes not in the Illumina TrueSight One panel would not be identified through this approach. 71.5% of this cohort remained without a genetic diagnosis even after EE panel testing, and evaluation by exome sequencing may lead to identification of previously unknown causative genetic variations or new candidate disease genes. Improved knowledge in clinical and genotypic registries, polygenic inheritance and recognition of specific contributing epigenetic factors may also reduce the number of unexplained cases [38,39].

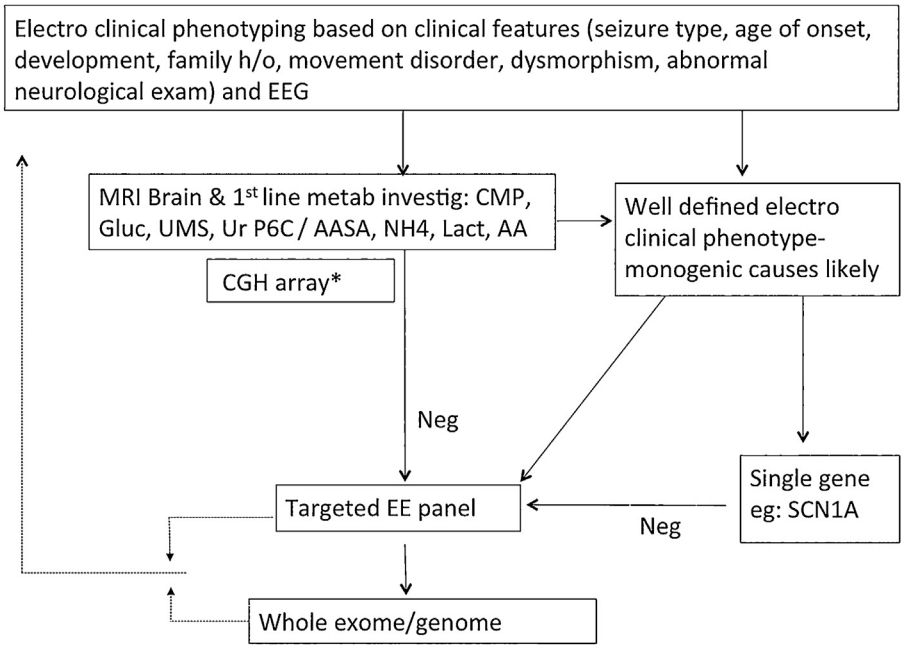

Fig. 3. Approach to genetic evaluation in children with epileptic encephalopathy. The flow chart describes the proposed approach to genetic testing in children with difficult to control epilepsy. This begins with careful electroclinical phenotyping prior to the evaluation which inturn is important for the interpretation of variants identified in the gene testing. The aim of the flowchart is to request basic metabolic investigations to look for treatable metabolic disorders and subsequent progression to EE panel testing rather than performing all second line metabolic investigations especially if MRI/CGH microarray are normal.
Abbreviations: EEG, electro encephalopgram; MRI, magnetic resonance imaging; CMP, calcium, magnesium, phosphorous; gluc, glucose; UMS, urine metabolic screen; Ur P6C, piperideine-6-carboxylate; AASA, α-aminoadipic semialdehyde; NH4, ammonia; Lact, lactate; AA, aminoacids; CGH array, comparative genomic hybridization microarray; Neg, negative.
*Vitamin trial to be considered in early onset refractory seizures; CSF studies to be considered where indicated.

In summary, the EE panel genetic testing translated to a diagnosis in 28.5% of patients (30 of 105) in our study. The early use of EE panel testing is economical and potentially circumvents the prolonged 'diagnostic odyssey' of the past, thereby changing the clinical pathway for investigation of these patients with refractory epilepsy. Careful clinical phenotyping improves the diagnostic yield, and allows economic use of the EE panel testing.

Author's contributions

KK designed study, performed literature review, clinical phenotyping, data analysis and drafted the paper under supervision of BB and DG. KH and EF helped with genetic data analysis & interpretation of the data and drafted the paper. All above authors discussed methodology, presentation of data, and edited the paper. JC/GH/ML helped initiate the Western Sydney Genomic project. JC/CT/SG/RD/RW/PP/MM/SAH/JA contributed clinical data and reviewed the manuscript.

Ethical publication statement

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.

Disclosure

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

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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.2018.05.005.

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