ARTICLE

Diagnostic Yield of Whole Genome Sequencing After Nondiagnostic Exome Sequencing or Gene Panel in Developmental and Epileptic Encephalopathies

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Abstract

Objective

To assess the benefits and limitations of whole genome sequencing (WGS) compared to exome sequencing (ES) or multigene panel (MGP) in the molecular diagnosis of developmental and epileptic encephalopathies (DEE).

Methods

We performed WGS of 30 comprehensively phenotyped DEE patient trios that were undiagnosed after first-tier testing, including chromosomal microarray and either research ES (n = 15) or diagnostic MGP (n = 15).

Results

Eight diagnoses were made in the 15 individuals who received prior ES (53%): 3 individuals had complex structural variants; 5 had ES-detectable variants, which now had additional evidence for pathogenicity. Eleven diagnoses were made in the 15 MGP-negative individuals (68%); the majority (n = 10) involved genes not included in the panel, particularly in individuals with postneonatal onset of seizures and those with more complex presentations including movement disorders, dysmorphic features, or multiorgan involvement. A total of 42% of diagnoses were autosomal recessive or X-chromosome linked.

Conclusion

WGS was able to improve diagnostic yield over ES primarily through the detection of complex structural variants (n = 3). The higher diagnostic yield was otherwise better attributed to the power of re-analysis rather than inherent advantages of the WGS platform. Additional research is required to assist in the assessment of pathogenicity of novel noncoding and complex structural variants and further improve diagnostic yield for patients with DEE and other neurogenetic disorders.

MORE ONLINE

Infographic
http://links.lww.com/WNL/B349

Podcast
Dr. Stacey Clardy talks with Dr. Elizabeth Emma Palmer about the diagnostic yield of whole genome sequencing after nondiagnostic exome sequencing or gene panel in developmental and epileptic encephalopathies.
NPub.org/54tdpf

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Go to Neurology.org/N for full disclosures. Funding information and disclosures deemed relevant by the authors, if any, are provided at the end of the article.

Glossary

ACMG = American College of Medical Genetics; **CMA** = chromosomal microarray; **DEE** = developmental and epileptic encephalopathy; **ES** = exome sequencing; **ILAE** = International League Against Epilepsy; **LCL** = lymphoblastoid cell line; **LRS** = long-read sequencing; **MGP** = multigene panel; **MPS** = massively parallel sequencing; **TAD** = topologically associated domain; **WGS** = whole genome sequencing.

Developmental and epileptic encephalopathies (DEE) encompass disorders in which epileptiform EEG abnormalities contribute to cognitive slowing/regression. They also include disorders in which developmental delay emerges before the presence of epileptic activity or in the presence of infrequent epileptic activity. Over 400 different monogenic causes for DEE have been reported and clinical overlap between these limits the possibility of identifying causes on clinical grounds. Identifying a molecular diagnosis leads to the possibility of directly guiding management and facilitates accurate genetic counseling and family planning.[1]

The recent advent of massively parallel sequencing (MPS) has changed the diagnostic landscape for DEE, resulting in diagnostic yields of 20%–40% for multigene panel (MGP) and 40%–60% for exome sequencing (ES), compared to <10% prior to MPS.[2] Whole genome sequencing (WGS) has the potential to improve diagnostic yields further, by improved coverage of exonic regions, ability to detect variants in noncoding regions and the mitochondrial genome, expansion variants (such as the expansion variant in *ARX*), alterations in methylation of DNA, as well as more robust detection of some structural variants, especially those with breakpoints in repetitive regions and copy-neutral variants such as inversions and translocations.[3] However, commercial diagnostic WGS is more costly and less accessible than ES or MGP.

There is no consensus position statement regarding the role of different types of MPS for DEE and a careful evaluation of the best approach for the diverse presentations of DEE is required. The objective of this study was to evaluate the diagnostic yield of WGS in patients with DEE, who had been uniformly investigated with metabolic screening, chromosomal microarray (CMA), and prior MPS testing (ES or MGP).

Methods

An overview of the study design is provided in figure 1.

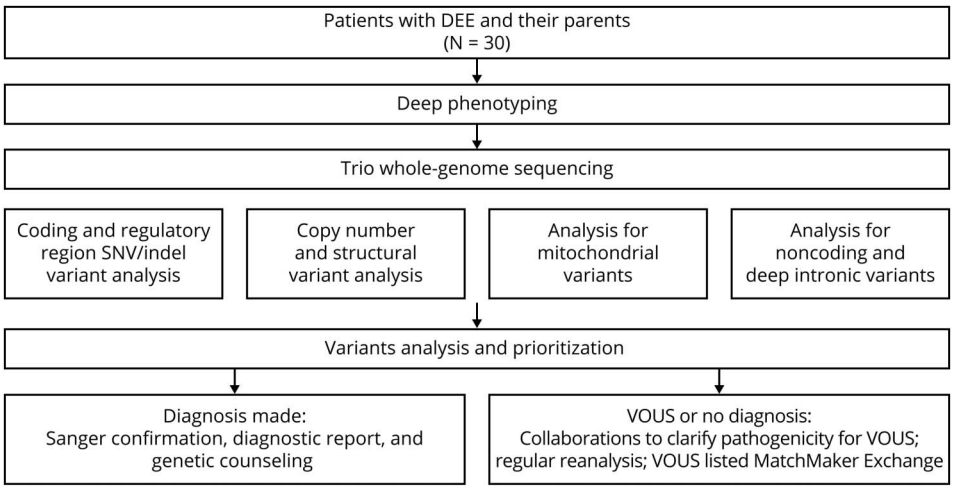
Standard Protocol Approvals, Registrations, and Patient Consents

The research was approved by the ethics committees of The Sydney Children's Hospital Network (LNR/13/SCHN/112) and the Prince of Wales Hospital Campus, Sydney, Australia (HREC ref 13/094). Clinical data were obtained from each affected individual's clinical team by analysis of the hospital medical records, with radiologic images and clinical photographs obtained as part of standard diagnostic procedures. Written informed consent was obtained from the participant's legal guardians for the conduct of genomic studies and for the publication of clinical and radiologic data and photographs.

Cohort Recruitment

All children who attended the Genetic Epilepsy Clinic of Sydney Children's Hospital, Randwick, between January 2017 and January 2018 and who met the following inclusion and exclusion criteria were recruited into the study. There were 2 subcohorts. Cohort A comprised 15 children who remained undiagnosed after completion of our prior trio ES study and whose families consented to proceed to WGS. Only one family did not consent to proceed to WGS. These children all had onset of seizures prior to 18 months of age and met the 2010 International League Against Epilepsy (ILAE) definition of epileptic encephalopathy,[4] namely (1) drug-resistant epilepsy for a minimum of 6 months, (2) seizure onset accompanied by adverse effect on development, such as developmental stagnation or regression, and (3) at least one EEG that was significantly abnormal with diffusely poorly organized background and marked bihemispheric epileptogenic activity. They also needed to have DNA and consent available from both parents to allow trio genetic testing. Affected individuals were excluded if they had a clear genetic or other etiologic diagnosis previously established on first-tier testing such as tuberous sclerosis (MIM: 605284), *SCN1A*-related Dravet syndrome (MIM 182389), a major structural/focal anomaly on neuroimaging, vascular stroke, head injury, infection, or ischemia. Subtle or generalized features on neuroimaging such as enlarged CSF spaces, nonspecific hyperintense lesions of 1–2 mm, or anatomical variants of normal structures such as the corpus callosum, cavum vergae, cisterna magna, or vascular variants did not preclude inclusion. Individuals were excluded if the primary neurologist or clinical geneticist was not in agreement with the enrollment of family in study, or if the patient was already entered into another research genetic study. Cohort B included 15 children undiagnosed after standard diagnostic testing, which over the time period of this study (2017–2019) included an MPS DEE panel (inclusive of 71 genes, described in detail in Kothur et al.[5]), CMA, metabolic screening, and, where there were clinically suggestive features, screening for expansions in *ARX* and metabolic and mitochondrial conditions. Clinical inclusion criteria were broadened to include children with (1) drug-resistant epilepsy (ongoing seizures despite trial of 2 anticonvulsants) for a minimum of 6 months, (2) effect on development: stagnation or regression, and (3) childhood

Figure 1 Flowchart of Whole Genome Sequencing Study



DEE = developmental and epileptic encephalopathy; SNV = single nucleotide variant; VOUS = variant of uncertain significance.

onset of seizures (<5 years of age). This reflected the updated 2017 ILAE definition of DEE.[6] Exclusion criteria were the same as for cohort A. Details of the phenotype of children in cohorts A and B with case descriptions of diagnosed patients including nondiagnostic testing completed prior to study enrolment are available in Dryad (case descriptions and table e-1, doi.org/10.5061/dryad.s1rn8pk67).

Whole Genome Sequencing

DNA from affected individuals and both unaffected parents was sequenced on an Illumina HiSeqX platform at the Kinghorn Centre for Clinical Genomics, Sydney, obtaining ~120 Gb data per sample, equivalent to >30 × average coverage, as previously described,[7] with all 30 samples joint called together. At this depth, >95% of coding exons were sequenced to >20× depth, which provided an adequate baseline for comprehensive variant characterization.

Data Filtering and Variant Prioritization for WGS

Variants were called using a GATK best practice analysis pipeline[8] and gVCF files from all individuals in the cohort were joint called in one batch to reduce the effect of confounding technical variables. Sequence variants were entered into the in-house platform SEAVE,[9] facilitating filtering and prioritization of variants. ClinSV (github.com/KCCG/ClinSV) was used to identify copy number and structural variants using a combination of discordantly mapped read pairs, split-mapping reads, and depth of coverage changes (article in preparation). ClinSV annotates variants using the ENSEMBL gene set v75[10] and previously reported structural variants from the Database of Genomic Variants.[11] ClinSV has a low false-positive rate (1.5%–4.5%) with high reproducibility (95%–99%) in detecting copy number variants from WGS data compared to clinical microarray and can also identify 4.7% of clinically reportable variants from a prospective clinical cohort (n = 485) within a diagnostic laboratory, of which 35%–63% were not detectable by current clinical CMA designs. The corresponding read-alignments were manually inspected using the Integrative Genomics Viewer (IGV).[12] Sequence variants were prioritized for analysis if they had a frequency <1% in the population databases EXAC (version 0.2), ESP (version 3/2013), and 1,000 Genomes (1000G Phase3 v20130502) and had a minimum variant quality >200 (determined by running GATK VQSR v3.3).

All trios were analyzed for the following inheritance patterns: de novo dominant, homozygous recessive, hemizygous, and compound heterozygous. In addition, for each family, sequence and structural variation were analyzed together, so that autosomal recessive conditions caused by a structural variant on one allele and a sequence variant on the other allele could be identified.

The *mity* analysis pipeline github.com/KCCG/mity was used to identify low heteroplasmy variants in the mitochondrial genome.[13] Mity was developed to specifically identify low (<1%) heteroplasmy single nucleotide polymorphisms and indels in the mitochondrial genome from a blood sample. Variants were analyzed by comparing the variant heteroplasmy in the proband to the parents and by comparison with population and disease databases, including MITOMAP[14] and the human mitochondrial genome database.[15]

The ROHmer analysis pipeline (Puttick et al., article in preparation) was used to identify regions of homozygosity, which assisted in prioritization of homozygous variants causal of autosomal recessive conditions in regions of homozygosity in the probands, and for analysis of other disease mechanisms such as uniparental disomy. Kinship coefficients were calculated to assess for the degree of parental relatedness using the

Table 1 Detected Variants, Phenotype, and Reason Not Diagnosed by Prior Exome Sequencing (ES) or Multigene Panel (MGP)

Cohort/ sex/age	Gene/chromosomal variant and inheritance	Phenotype: age at onset/seizure description/EEG/ level ID/other (extra) neurologic features	Reason not reported by ES study (cohort A) or by diagnostic MGP (cohort B)
A1/F/7 y	NM_001020658.1 (*PUM1*): c.3439C > T de novo	4 mo/DRE (focal)/MEA/profound ID/ptosis + dysmorphism	New gene now linked to DEE
A2/M/6 y	NM_024678.5 (*NARS2*): c.[167A > G]; [749G > A]	5 mo/DRE (focal)/MEA/profound ID/movement disorder, ptosis	More support for pathogenicity
A3/M/4 y	Chr5 (GRCh37): 88216182–97291426 inv de novo	11 mo/DRE (multiple)/MEA/moderate ID/ASD	Copy neutral inversion undetectable by ES/CMA
A4/M/4 y	NM_006772.2(*SYNGAP1*): c.85_ 86delAT p.(Met29AlafsTer11) hom	10 mo/DRE (multiple)/MEA/profound ID, movement disorder	More support for pathogenicity
A5/M/6 y	ChrX (GRCh37): 73565151–73956350 dup (mat)	6 mo/EpS (+multiple)/hyps/severe ID, movement disorder	Complex inverted duplication undetectable by ES/CMA
A6/F/10 y	NM_003165.3 (*STXBP1*): c.[998_ 1000delAGA] de novo	4 mo/EpS (+multiple)/MEA/severe ID/ASD	More support for pathogenicity
A7/F/3 y	NM_021032.4 (*FGF12*): c.341G > A de novo	Neonatal/tonic seizures/MEA/profound ID	New gene now linked to DEE
A8/F/4 y	ChrX (GRCh37): 62821285–62868236 inv de novo	6 mo/EpS/MEA/moderate ID and ASD	Copy neutral inversion not detectable by ES/CMA
B1/F/3 y	NM_003165.3 (*STXBP1*): c.59_ 62delAGAA de novo	Neonatal/DRE (multiple)/MEA/severe ID/microcephaly	Gene not detected (technical error)
B2/M/4 y	NM_015284.3 (*SZT2*): c.[6007delG]; [5734C > T]	3 mo/EpS (+multiple)/MEA/moderate ID/dysmorphism	Gene excluded MGP
B3/M/6 y	NM_021008.3 (*DEAF1*): c.646A > G de novo	<3 y/DRE (multiple)/MEA/severe ID/ASD	Gene excluded MGP
B4/F/14 y	NM_003042.3 (*SLC6A1*): c.187G > A de novo	9 mo/DRE (multiple): focal EEG/severe ID/ASD	Gene excluded MGP
B5/M/3 y	NM_024818.4 (*UBA5*): c.692C > T hom	4 mo/EpS (+multiple)/hyps/severe ID/microcephaly	Gene excluded MGP
B6/F/3 mo	NM_152296.4 (*ATP1A3*): c.875T > G de novo	Neonatal DEE/MEA/PMG/microcephaly	Gene excluded MGP
B7/F/5 y	NM_001007026.1 (*ATN1*): c.3188T > G de novo	7 wk/DRE (multiple): MEA/dysmorphism	Gene excluded MGP
B8/F/15 y	NM_015559.3 (*SETBP1*): c.2885_ 2887delCCA de novo	18 mo/DRE (multiple)/MEA/severe ID/ptosis, cleft palate	Gene excluded MGP
B9/F/16 y	NM_019109.4 (*ALG1*): c.[773C > T]; [1,187+3A > G]	3 mo/DRE (multiple)/hyps/severe ID/movement disorder, dysmorphism	Gene excluded MGP
B10/M/ 13 y	NM_003896.3 (*ST3GAL5*): c.[254T > G]; [1229T > C]	8 mo/DRE (multiple)/MEA/severe ID/movement disorder/SNHL	Gene excluded MGP
B11/F/8 mo	NM_007077.4 (*AP4S1*): c.139-2A > G hom	7 mo/DRE (multiple)/focal EEG/severe ID/microcephaly	Gene excluded MGP

Abbreviations: ASD = autism spectrum disorder; CMA = chromosomal microarray; DEE = developmental and epileptic encephalopathy; DRE = drug-resistant epilepsy; EpS = epileptic spasms; EpS (+multiple); epileptic spasms evolving to multiple seizure types; (focal) = focal onset; hom = homozygous; hyps = hypsarrhythmia; ID = intellectual disability; mat = maternal; MEA = multifocal epileptogenic activity on EEG; (multiple) = multiple seizure types; PMG = polymicrogyria; SNHL = sensorineural hearing loss; WGS = whole genome sequencing.

pipeline KING[16] to confirm sample identity and exclusion of nonpaternity.

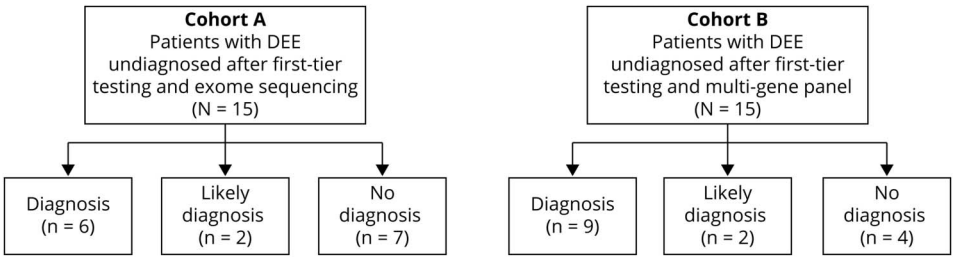
Assessment of Pathogenicity of Filtered Detected Variants

Assessment of the potential pathogenicity of candidate variants followed international guidelines[17] and as previously reported.[2] Further analysis of pathogenicity, when appropriate, required a combination of segregation studies in the extended family and functional studies.

Sanger Confirmation and Reporting of Variants

Variants classified as pathogenic or likely pathogenic were submitted to a diagnostic laboratory for independent bidirectional

Figure 2 Results of Whole Genome Sequencing Study



DEE = developmental and epileptic encephalopathy.

Sanger sequencing confirmation and the issuance of a diagnostic report. Results were returned to the family by their respective clinical geneticist and neurologist.

Functional Validation of Novel Variants

When a novel DEE gene or candidate gene variant was identified, collaborations were established to clarify pathogenicity. This included entry of the variant in the MatchMaker exchange hub[18] and contacting basic science researchers. Fibroblast or lymphoblastoid cell lines (LCLs)[19] were generated from the patient and parents using standard methodology when required for functional studies. The interstitial chromosome inversion for individual 3 was validated using inversion PCR using previously described methodology.[20] Primers were designed within unique regions flanking the predicted inversion breakpoints and different primer combinations were used to detect the presence of DNA in specific orientations (for additional detail on methods, see data available from Dryad, doi.org/10.5061/dryad.s1rn8pk67). Hi-C performed in LCLs from individual 3 was processed following standard protocols.[21] Libraries were sequenced for 320 million fragments in a 75 bp paired-end run on a HiSeq4000 (Illumina) and the Hi-C map was generated by pooling 4 technical replicates. Hi-C data was processed using Juicer pipeline,[22] with the Hi-C map (raw count map) created using read-pairs with MAPQ30 or above and visualized using Juicebox.[23] As a control, we used data from one nonaffected participant's LCLs, described elsewhere.[24]

Data Availability

All data on detected genomic variants considered causal or likely causal of the affected individual's phenotype are available as open access variants in the public repository DECIPHER (accession numbers 317034, 317297, 357719, 359420, 349687, 385396, 369157, 355952, 351637, 385397, 351462, 345520, 353800, 385491, 362041, 362038, 371181, 380517).

Results

In cohort A, an additional 6 diagnoses meeting American College of Medical Genetics (ACMG) criteria class IV or V[17] and an additional 2 likely diagnoses were made, resulting in an overall diagnostic yield of 53% (8/15). Likely diagnoses

Figure 3 Clinical Photographs of the Proband With de Novo Inversion Adjacent to *MEF2C*

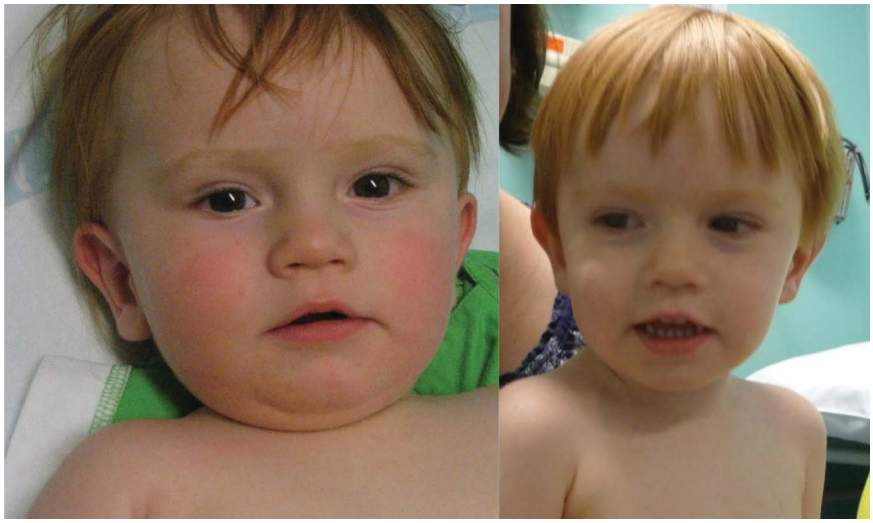
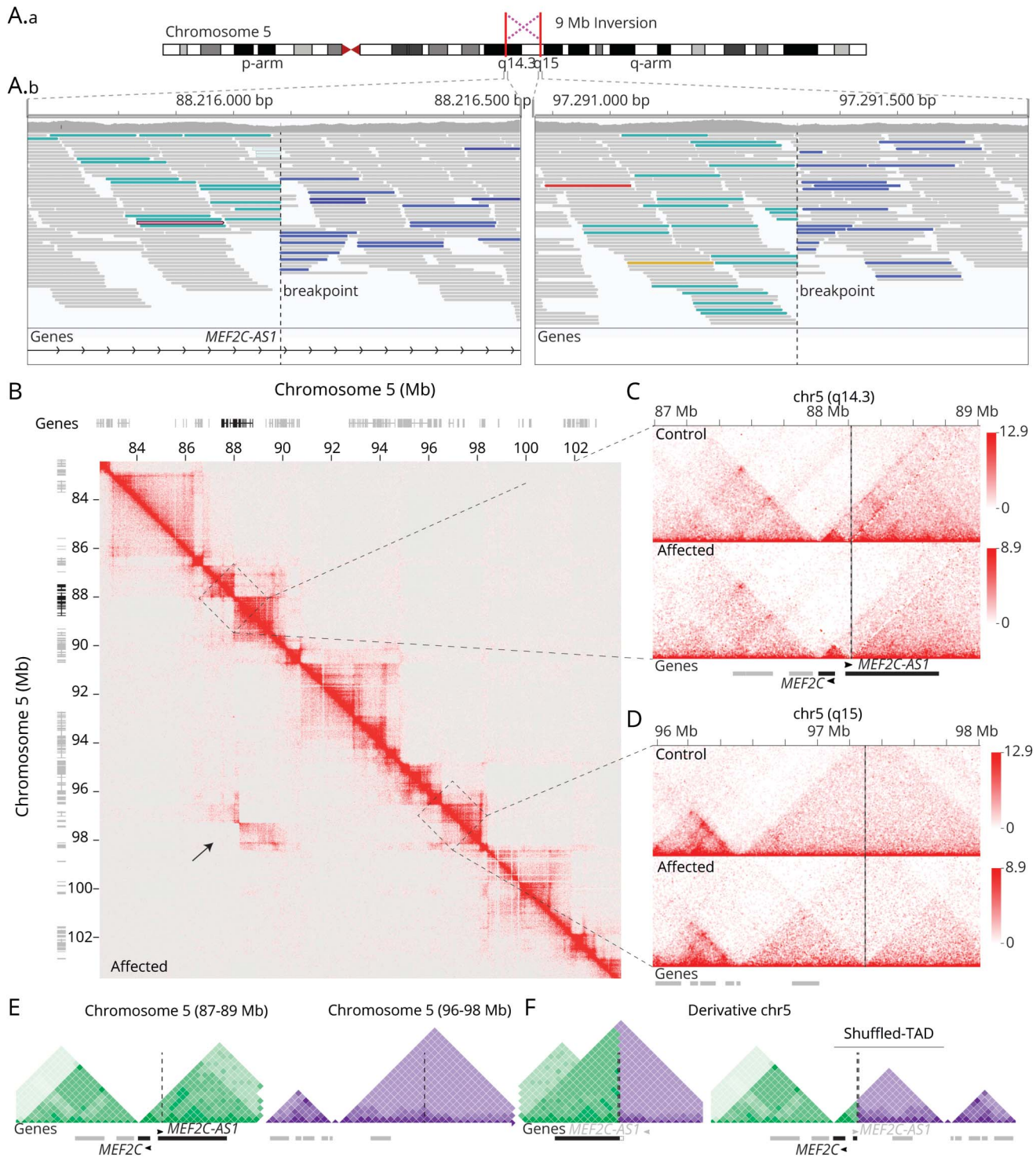


Figure 4 Molecular (Whole Genome Sequencing and Hi-C) Features of Individual 3



(A.a) Position and size of inversion on chromosome 5. (A.b) Screenshots from integrated genome viewer and the split reads and discordant pairs identifying a copy neutral inversion 16Kb upstream of the first exon of *MEF2C*. (B) Hi-C performed in patient-derived lymphoblastoid cell line shows ectopic chromatin interaction in a bowtie pattern (arrow), which indicates inversion that results in a shuffled topologically associated domain (TAD) (25 kb resolution; raw count map). Black squares with dashed lines indicate both chromosome regions involved in this inversion. *MEF2C* and its antisense long noncoding RNA are represented by dark lines and bars. Other genes are represented by gray bars. (C) Zoom-in on the chr5 (q14.3) region. The inversion breakpoint is shown by vertical dashed line (10 kb resolution; KR normalized). (D) Hi-C cis-map of the chr5 (q15) region shows loss of chromatin contact in the affected map caused by the inversion (10 kb resolution; KR normalized). (E) Schematic representation of TAD structures on the wild-type chromosome 5. (F) Schematics of the derivative chr5 show a shuffled TAD allowing the contact of *MEF2C* promoter with enhancers from chr5 (q15) region.

Table 2 Genetic Conditions Diagnosed by Exome Sequencing or Whole Genome Sequencing, Grouped by Molecular Pathway: Inheritance and Clinical Utility

Genetic diagnosis, inheritance, and OMIM reference (MIM number)	Inheritance	Effect of diagnosis on management	Effect of diagnosis for family
Metabolite transporters (n = 1)			
De novo *SLC6A1* encephalopathy (MIM 616421)	AD		EDO, FC, GF, RC
Transcription, DNA repair, and chromatin remodeling (n = 5)			
De novo *SETBP1* encephalopathy (MIM 616078)	AD		EDO, FC
De novo chromosomal inversion upstream *MEF2C*	AD		FC
De novo *PUM1* encephalopathy (MIM 617931)	AD		ASG, EDO, FC
De novo *ATN1* encephalopathy (awaiting MIM)	AD		ASG, FC, EDO, RC
De novo *DEAF1* encephalopathy (MIM 15828)	AD		ASG, FC, EDO
Protein translation and modification (n = 5)			
***NARS2* encephalopathy (MIM 616239)**	AR	*	FC, RC
***UBA5* encephalopathy (MIM 617132)**	AR		EDO, RC
***RLIM* duplication syndrome (awaiting MIM)**	XL		ASG, EDO, FC, RC
De novo *STXBP1* encephalopathy (MIM 612164)	AD		ASG, FC, RC
De novo *FGF12* encephalopathy (MIM 617166)	AD		EDO, FC
Postsynaptic signalling (n = 4)			
De novo ARHGEF9 encephalopathy (MIM 300607)	AD		EDO, FC
***SYNGAP1* encephalopathy (MIM 612621)**	AR	GHS	EDO, FC
De novo *ATP1A3* encephalopathy (awaiting MIM)	AD	*	FC, RC
***SZT2* encephalopathy (MIM 615476)**	AR	GAD2†	FC, RC
Other metabolic (n = 3)			
***ALG1* encephalopathy (MIM 608540)**	AR		ASG, EDO, FC
***ST3GAL5* encephalopathy (MIM 609056)**	AR	*	EDO, FC
***AP4S1* encephalopathy (MIM 614067)**	AR		ASG, EDO, FC

* Individual deceased before genetic diagnosis reached.
† Trial of rapamycin (targeted therapy).
Abbreviations: AD = autosomal dominant; AR = autosomal recessive; ASG = access to support groups/information; AI = avoided investigations; EDO = end diagnostic odyssey; FC = family closure; GAD = guidance on antiepileptic drug; GHS = guidance on health surveillance; GF = improved government funding; PC = palliative care; RC = reproductive counseling; XL = X-linked.

included variants that were assessed as likely pathogenic based on the ACMG framework and additional functional studies. Of these, 20% (3/15) were unique diagnoses related solely to WGS. These were copy neutral or complex structural variants that were not (fully) detected by ES or first-tier testing (including CMA). Five individuals had variants in genes that were detectable on ES, but that did not, at the time of conclusion of the ES study, have enough supportive clinical or functional data to support a (likely) pathogenic diagnosis (table 1). In cohort B, 9 definite and 2 likely additional diagnoses were made (11/15 [73%]). The majority (n = 10) had variants in genes not included in the MPS gene panel previously used for the affected individuals. One variant was missed by the panel for technical reasons. A summary of the genomic results in this cohort is provided in figure 2 and table 1. Overall, 8 of the diagnosed individuals (8/19: 42%) had variants that were inherited in an X-linked or autosomal recessive manner. Detail of the variants assessed as (likely) pathogenic is provided in Dryad (table e-1, doi.org/10.5061/dryad.s1rn8pk67). The structural variants detected in cohort A are described in more detail below, as these case reports highlight the benefit of WGS in the detection of complex structural variation.

Case Reports of Affected Individuals With Structural Variants Delineated by WGS

Individual A8

A 4-year-old girl with onset of epileptic spasms at 6 months, evolving to multifocal epileptiform activity, intellectual

disability, and autistic features, had a copy neutral inversion on the X chromosome detected by WGS. Breakpoints were able to be precisely defined (ChrX:62821285–62868236) to demonstrate that one breakpoint lay within the DEE and autism gene *ARHGEF9*. The family did not provide consent for patient-derived cells to assess *ARHGEF9* expression, but the patient's phenotype matched that of other individuals with *ARHGEF9*-related encephalopathy (epileptic encephalopathy, early infantile, 8 MIM 300607). In addition, another female patient has been described with a severe neurocognitive phenotype and a de novo chromosomal inversion disrupting *ARHGEF9*, who had reduced *ARHGEF9* expression.[25]

Individual A5

A 5-year-old boy with onset of epileptic spasms at 6 months with a hysparrhythmic EEG, evolving to multiple other seizure types, severe intellectual disability, and a movement disorder had previously had a maternally inherited Xq13 chromosomal duplication of uncertain significance detected by CMA. WGS revealed a more complex duplication-normal-duplication rearrangement whereby one of the duplicated sections was shown to be inverted, and refined the breakpoints of the second duplication (ChrX:73565151–73956350), enabling demonstration that one of the breakpoints lay within a well-established DEE gene *NEXMIF* (previously known as *KIAA2022*).[26] This patient and the molecular and protein effects of this complex structural rearrangement are described in more detail elsewhere, in which we delineate a neurocognitive phenotype in association with Xq13 duplications in 8 individuals, all of whom have consistently increased RLIM protein and mRNA levels, and varied in their inclusion of neighboring genes including *NEXMIF*.[27]

Individual A3

A 4-year-old boy with infantile hypotonia, drug-resistant epilepsy (atonic seizures diagnosed at 9 months evolving to multiple seizure types), severe intellectual disability and absent speech, autism, stereotypical hand flapping movements, a broad-based gait, and no severe dysmorphism (figure 3) remained undiagnosed after extensive neurometabolic testing and trio exome sequencing. He proceeded to WGS. No candidate sequence variants in the nuclear or mitochondrial DNA were identified. Analysis for structural variants using ClinSV identified a 9 Mb balanced de novo chromosomal inversion with a breakpoint 16 kb upstream of the known neurodevelopmental and DEE gene *MEF2C*, which would be predicted to transect the long noncoding RNA gene *MEF2C-AS1* (figure 4A). CMA was unable to detect this variant as it was copy number neutral and this variant would not have been detected by ES as the proximal breakpoint was in a noncoding region.

This de novo inversion was further evaluated for causality, even though it did not directly transect a known DEE gene, because other de novo chromosomal events, including translocations, inversions, and deletions, in the region upstream of *MEF2C* had been previously shown to result in a severe neurodevelopmental disorder comparable to *MEF2C*-related encephalopathy.[28,29] Indeed, our proband had a strikingly similar phenotype to *MEF2C* encephalopathy, which is characterized by severe intellectual disability with absent speech, seizures, hypotonia, autistic features, and stereotypical movements.[30]

It has been postulated that structural events adjacent to *MEF2C* disrupt the control of *MEF2C* expression in the developing brain. One possible explanation for this could be if a structural variant disrupts the local topologically associated domain (TAD). A TAD is a region of DNA, typically on a megabase scale, that has been shown to contain multiple loci that interact with each other at high frequency, but infrequently interact with loci in the rest of the genome. TADs are understood to represent fundamental functional units of the genome and recently, structural variants that disrupt TADs around a critical gene have been implicated in a variety of human conditions, including neurologic disorders.[31] *MEF2C* is contained within a >2 Mb TAD, along with 6 other genes. We first were able to confirm the inversion using another method, inversion PCR (figure e-1, data available from Dryad). There was no evidence of constitutional epimutation at the MEF2C CpG island promoter (figure e-2, data available from Dryad).

We proceeded to interrogate the effect of this inversion on the local TAD structure using the chromosome conformation capture technique Hi-C and were able to demonstrate that the inversion results in a shuffling TAD in LCL from the proband (figure 4). This shuffling TAD means that some critical regulatory elements for *MEF2C* are likely now placed in a separate TAD from the *MEF2C* gene itself, affecting *MEF2C* expression in certain tissues. Despite this evidence, as is the case for several other patients with de novo variants in this region and a neurocognitive phenotype,[28,29] we were unable to demonstrate that expression of *MEF2C* or its interactors *MECP2* or *CDKL5* was significantly reduced in the clinically accessible tissue of LCLs, compared to LCLs from the unaffected father, who does not have the inversion (data available from Dryad, figure e-3, doi.org/10.5061/dryad.s1rn8pk67). The possible reasons for this are discussed in the following.

Demographic and Phenotypic Characteristics of Diagnosed Patients

The demographic and phenotypic characteristics of the 19 affected individuals who received a diagnosis were compared with those of the 11 individuals remaining undiagnosed at the end of the study, to determine whether any patterns emerged, using Fisher exact test. No statistically significant differences emerged (data available from Dryad, table e-2, doi.org/10.5061/dryad.s1rn8pk67).

Discussion

This study examines the diagnostic yield of WGS in patients with DEE previously undiagnosed by a pathway that included

CMA, metabolic screening, and MPS: either an unbiased ES (cohort A) or MGP approach, which applied a gene panel focused on previously reported epileptic encephalopathy genes (cohort B). Diagnostic yield was improved in both cohorts, with 8 additional diagnoses made in cohort A (8/15; 53%) and 11 additional diagnoses made in cohort B (11/15; 73%).

For cohort A (patients undiagnosed by our prior ES study), diagnostic yield was improved in a third of cases (5/15) secondary to alteration in the assessment of pathogenicity of variants detectable by ES but that did not, at the time of completion of that study, have sufficient evidence to suggest causality. Thus, these additional diagnoses are more appropriately attributed to the power of MPS reanalysis rather than any inherent superiority of WGS over ES. This is consistent with recent reports for other Mendelian conditions, which demonstrate the value of reanalysis of unbiased genomic data.[32] For DEE, the Epilepsy Genetics Initiative[33] recently reported that 8/139 (5.8%) ES-negative patients attained a putative diagnosis due to systematic reanalysis of the ES data. Reanalysis allows for consideration of evidence provided by new publications that associate a new gene with DEE or lend critical support for pathogenicity to a variant in a known DEE gene (see table 1). Reanalysis can also be facilitated by the new listing of recurrent variants in affected individuals with overlapping phenotypes in public variant databases, such as ClinVAR or DECIPHER.[34] Reanalysis has been consistently shown to improve diagnostic yield and is a strong argument in favor of first using an unbiased MPS approach (ES or indeed WGS) rather than a biased gene panel, to allow for later detection of variants in novel DEE genes.

WGS was able to improve diagnostic yield in cohort A by delineation of 3 complex structural variants that were undetectable on prior ES or MGP and CMA (3/30 [10%]). This benefit of WGS for DEE was also demonstrated by the only other WGS study for DEE in which affected individuals had been comprehensively prescreened by MGP and CMA.[35] WGS allows identification of structural events not detected by cytogenetic methods, including CMA, due to their size or because they are balanced with respect to copy number or act by position effects.[3] Interpretation of the clinical significance of complex structural variants will benefit from improved understanding of the effect of variants on 3D genomic structure, as the case study (individual 3) in this article illustrates.

For patients in cohort B (undiagnosed by MGP), the diagnostic yield was 73% (11/15). All but 1 of the additional 11 diagnoses in this group (10/15 [67%]) could not have been made by the locally available gene panel, as the relevant gene was not included in the panel at the time the patient was tested.[5] This speaks to the inherent limitation of gene panels, which cannot keep pace with the explosion of gene discovery in neurocognitive disorders such as DEE.

Our study did not find evidence for pathogenic mitochondrial or noncoding sequence variants in either cohort, as was also the case for the 3 prior WGS studies in DEE.[35–37] Although mitochondrial variants are detectable by WGS, how best to provide evidence for pathogenicity of such variants is still an area of active research, and moreover patients with a phenotype suggestive of a mitochondrial condition are routinely prescreened in our institution by specific mitochondrial disorder screening (data available from Dryad, case descriptions, doi.org/10.5061/dryad.s1rn8pk67).

To date, only 3 studies of the effect of WGS for the investigation of DEE have been published.[35–37] In the largest study, WGS was applied to a cohort of 197 patients with DEE who remained undiagnosed after standard clinical diagnostic testing (MGP and CMA) and a molecular diagnosis was established in 32% of individuals.[35] Of those, 84% had de novo sequence variants, 10% had autosomal or X-linked recessive sequence variants, and 6% had de novo copy number variants (4/63 cases solved). A second study[36] of 14 patients reported a 100% yield for WGS. In this study, prior genetic testing was limited: only 21% had undergone a MGP and none had ES. The authors discuss that 86% of the diagnoses would have been ascertained by MGP or ES, but an additional 2 structural variants (2/14 [14%]) that were detected would have been missed by ES. Thus, this study reaches similar conclusions to the current study. The third study also achieved a 100% molecular diagnosis for 6 patients tested by WGS, all of whom had coding or canonical splicing variants that would have been diagnosed by ES.[37] Thus, although numbers were limited, our study extends and adds to the experience of the field by providing data from a different medical system, and by describing in detail the potential of WGS to improve detection and delineation of complex structural variants in DEE, as well as the challenges in proving pathogenicity of such variants.

The optimal way to detect structural variants by MPS is under debate. Short-read (150–300 bp) paired-end sequencing, such as the Illumina sequencing used in this study, with read depths of 30–40×, is considered by many to offer a good combination of sensitivity, accuracy, and price. However, the human genome is highly complex, with many long, repetitive elements, copy number, and structural variations, and many of these complex elements are so long that structural variants affecting such regions can be difficult to resolve with short-read paired-end technology.[38] To circumvent this difficulty, alternative technologies and approaches have emerged.

First, long-read sequencing (LRS) technologies have been developed that produce reads often >10 kb in size, with the potential to span complex and repetitive regions in a single continuous read.[39] LRS technologies offer improved detection and characterization of large structural variation, especially when the breakpoints are in highly repetitive regions of the genome. LRS technologies generally have higher error rates or higher costs of sequencing than short read technologies, meaning that they do not present viable alternatives for routine diagnostic testing. It is likely that this will change with further developments in LRS platforms.

Second, there have been efforts to overcome some of the limitations of short-read technology by using paired end mapping (or mate pair) sequencing,[40] or by using linked read technology.[29] Although both paired-end and linked-read sequencing have the potential to better resolve individual structural variants >1 kb in size or that have breakpoints within repetitive genomic regions, they both have currently lower sequencing yield and more uneven coverage than standard paired-end sequencing, likely resulting in a lower overall diagnostic yield.

An important take-home message is that DEE can frequently have a germline genetic cause identified. This study reached a (likely) diagnosis in 63% (19/30). The cumulative diagnostic rate in the cohort, subjected first to ES[2] followed by WGS in this study, was 73% (14 from ES study +8 from WGS study/30 originally enrolled candidates). We appreciate that this is a higher diagnostic rate compared to previous studies conducted in diagnostic laboratory settings. Both this and the prior study[2] were conducted in a rigorous research context, with careful follow-up of variants of uncertain significance and genes of uncertain significance with national and international clinical and basic scientific collaborators, contributing to delineation of novel genes and variants causative of DEE.[41–44] We acknowledge that a lower diagnostic rate would be anticipated through a diagnostic laboratory, but our work contributes to evidence supporting the importance of a pathway for a diagnostic–research interface such as an undiagnosed disease program to maximize diagnostic yield for children with rare genetic disorders with pressing need for genetic diagnoses.[45]

The causal genetic conditions in this study are shown in table 2, which groups these by molecular subtype. It is notable that expanding from MGP or ES to WGS did not identify any additional ion channelopathies, consistent with these disorders being well covered by MGP. In contrast, variants in genes implicated in basic and ubiquitous cellular functioning were commonly identified, particularly in individuals with onset of seizures after the neonatal period, and those with more complex presentations including movement disorders, dysmorphic features, or multiorgan involvement. This finding is noteworthy, and replicates findings of other recent studies that used an unbiased ES or WGS approach.[35,46,47] This reflects the overlap in genetic causes for DEE with other neurodevelopmental disorders, such as intellectual disability without epilepsy. The ILAE recommendation to change the term for this group of conditions from epileptic encephalopathy to developmental and epileptic encephalopathy (DEE) acknowledges this change in understanding the breadth of causes of severe early-onset epilepsies.[6]

A total of 42% (8/19) of the molecular diagnoses in this cohort were autosomal recessive or X-linked recessive conditions. That a sizeable proportion of DEE is due to autosomal recessive or X-linked conditions is being increasingly recognized by studies using ES or WGS[46,47] and has important genetic counseling implications. A total of 42% of the 19 diagnosed families have used the genetic test result in decision-making regarding a subsequent pregnancy or specifically for prenatal diagnosis (table 2).

We attempted to analyze whether any phenotypic or demographic features were significantly associated with a greater chance of reaching a diagnosis (data available from Dryad, table e-2, doi.org/10.5061/dryad.s1rn8pk67), but none were able to be identified. Although not reaching statistical significance, it should be noted that all affected individuals from consanguineous families (n = 3), with a neonatal onset to their seizures (n = 3), and with accompanying movement disorders (n = 5) were able to reach a diagnosis. Only 1 out of 6 individuals with dysmorphic features or evidence of multisystem involvement remained undiagnosed by the end of the study.

Although diagnostic yield is clearly superior for unbiased ES or WGS over MGP, we appreciate this is not the only factor a clinician considers when choosing the most appropriate diagnostic tests for each patient. MGP are currently often less expensive, and may have a faster turnaround, but have a lower chance of detecting a variant of uncertain significance or incidental finding.[48] Moreover, in neonatal onset seizures, MGP have a very similar diagnostic yield to ES, which likely reflects the high proportion of ion channelopathies and synaptic disorders in this subgroup.[5] MGPs are likely to become less commonly used testing methodologies as workflows for ES and WGS become more automated and their costs continue to fall. One reasonable approach is to order a gene panel on an exome backbone that could be reflexed to an ES if no diagnosis is made on the initial panel. This offers the advantage of speed of analysis, critical now that targeted therapeutics for DEE are available or on the imminent horizon, as well as potential cost-effectiveness.

With these considerations in mind, a pragmatic diagnostic pathway for DEE is proposed. Patients with a new-onset DEE should receive an urgent MPS test, in addition to CMA and metabolic screening, full blood count/blood smears, and hepatic and renal function testing. We recommend the inclusion of metabolic screening for newly presenting patients for 2 main reasons. First, these tests can still screen for important treatable causes of DEE more rapidly than most MPS testing.[49] Second, they can provide clarification of the pathogenicity for variants of uncertain clinical significance detected in inborn error of metabolism genes, for example alkaline phosphatase levels in patients with some disorders of the GPI (glycosylphosphatidylinositol) pathway[50] and abnormal full blood counts/blood smears in patients with uridine-responsive epilepsy due to pathogenic variants in *CAD*.[51] Screening for expansions in exon 2 of *ARX* and mitochondrial DNA testing should be included if MGP or ES is used as the MPS test, or WGS is used but the reporting of expansion and mitochondrial DNA variants is not included. Older patients with DEE who either were not or could not be diagnosed at disease onset could still be referred for a CMA and cost-effective MGP (reflexed to exome) and, if needed subsequently, evaluation by ES or WGS. The choice of MPS: gene

panel on an ES background, ES, or WGS will be dictated by local availability of tests, timeline for receiving results, and the funding model in use. It is important to recognize, as we have previously highlighted,[2] that the quality of MPS panels varies considerably depending on the quality of gene curation, and that the use of gene panels with larger numbers of genes included do not necessarily result in a higher diagnostic yield.[48]

DEE are a group of conditions with complex morbidity and high mortality. For the proportion of DEE due to an underlying genetic cause, identification of a molecular diagnosis is important in guiding a targeted therapeutic and management approach for the affected child and appropriate genetic counselling and support for the family.[1] Thus, there is a pressing need to understand how best to reach a timely molecular diagnosis for as many children with DEE as possible. Our study shows that WGS can improve the diagnostic yield for DEE over an ES or MGP approach, particularly for individuals with onset of seizures after the neonatal period or with complex neurologic or multiorgan presentations. Future research aiding the detection by WGS and assessment of pathogenicity of mosaic, noncoding, mitochondrial, expansion, and complex structural variants, including the addition of RNA sequencing to assist in the interpretation of variants putatively affecting splicing and an evaluation of alterations in genome-wide methylation, will be required to maximize the diagnostic yield in DEE and other, largely monogenic, neurocognitive conditions. Such research will also allow WGS to truly become a one-stop diagnostic test[3] and place patients with DEE in the best possible position to obtain a diagnosis, making them precision medicine ready.[1]

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Disclosure

The authors report no disclosures relevant to the manuscript. Go to Neurology.org/N for full disclosures.

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Rebecca Macintosh	Sydney, Australia	Delineated phenotype on included participants, reviewed and approved the manuscript
Uirá Souto Melo	Berlin, Germany	Design and conception of Hi-C studies, analyzed the data, reviewed and approved the manuscript
Stefan Mundlos	Berlin, Germany	Design and conception of Hi-C studies, analyzed the data, reviewed and approved the manuscript
Sarah Righetti	Sydney, Australia	Reviewed and edited the manuscript, prepared manuscript, tables and figures for submission
Tejaswi Kandula	Sydney, Australia	Delineated phenotype on included participants, reviewed and approved the manuscript
Andre E. Minoche	Sydney, Australia	Design and conception of ClinSV structural variant analysis tool, performed analysis of structural data from whole genome sequencing, reviewed and approved the manuscript
Clare Puttick	Sydney, Australia	Design and conception of ROHmer and *mity* analysis tools, performed analysis of mitochondrial data from whole genome sequencing, reviewed and approved the manuscript
Velimir Gayevskiy	Sydney, Australia	Design and conception of variant analysis platform SEAVE and Introme tools used to analyze whole genome sequencing data, reviewed and approved the manuscript
Luke Hesson	Sydney, Australia	Design and conception of inversion PCR analysis, reviewed and approved the manuscript
Senel Idrisoglu	Sydney, Australia	Design and conception of inversion PCR studies and data analysis, reviewed and approved the manuscript
Cheryl Shoubridge	Adelaide, Australia	Design and conception of LCL expression studies and performed data analysis, reviewed and approved the manuscript
Monica Hong Ngoc Thai	Adelaide, Australia	Design and conception of LCL expression studies and performed data analysis, reviewed and approved the manuscript
Ryan L. Davis	Sydney, Australia	Design and conception and data analysis of intronic analysis using Introme, reviewed and approved the manuscript
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Appendix *(continued)*

Name	Location	Contributions
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Mark J. Cowley	Sydney, Australia	Contributed to study design, conceptualized ClinSV, *mity*, ROHmer, SEAVE, and Introme data analysis tools, oversaw bioinformatics, reviewed and approved the manuscript
Tony Roscioli	Sydney, Australia	Chief Investigator on OHMR study funding grant, contributed to study design, obtained IRB consent for study, reviewed and approved the manuscript
Ann Bye	Sydney, Australia	Contributed to study design, delineated phenotype on included participants, reviewed and approved the manuscript
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