Genome sequencing identifies rare tandem repeat expansions and copy number variants in Lennox–Gastaut syndrome

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Epilepsies are a group of common neurological disorders with a substantial genetic basis. Despite this, the molecular diagnosis of epilepsies remains challenging due to its heterogeneity. Studies utilizing whole-genome sequencing may provide additional insights into genetic causes of epilepsies of unknown aetiology. Whole-genome sequencing was used to evaluate a cohort of adults with unexplained developmental and epileptic encephalopathies (n = 30), for whom prior genetic tests, including whole-exome sequencing in some cases, were negative or inconclusive. Rare single nucleotide variants, insertions/deletions, copy number variants and tandem repeat expansions were analysed. Seven pathogenic or likely pathogenic single nucleotide variants, and two pathogenic deleterious copy number variants were identified in nine patients (32.1% of the cohort). One of the copy number variants, identified in a patient with Lennox–Gastaut syndrome, was too small to be detected by chromosomal microarray techniques. We also identified two tandem repeat expansions with clinical implications in two other patients with Lennox–Gastaut syndrome: a CGG repeat expansion in the 5′ untranslated region of DIP2B, and a CTG expansion in ATXN8OS (previously implicated in spinocerebellar ataxia type 8). Three patients had KCNA2 pathogenic variants. One of them died of sudden unexpected death in epilepsy. The other two patients had, in addition to a KCNA2 variant, a second de novo variant impacting potential epilepsy-relevant genes (KCNIP4 and UBR5). Overall, whole-genome sequencing provided a genetic explanation in 32.1% of the total cohort. This study demonstrates that using whole-genome sequencing, the examination of multiple types of rare genetic variation, including those found in the non-coding region of the genome, can help resolve unexplained epilepsies.

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Abbreviations: ACMG = American College of Medical Genetics and Genomics; ASD = autism spectrum disorder; BAFME = benign familial adult myoclonic epilepsy; bp = base pair; c. = coding DNA change; Chr = chromosome; CMA = chromosomal microarray; CNV = copy number variant; DD = developmental delay; DEE = developmental epileptic encephalopathy; DR = developmental regression; GATK = Genome Analysis Toolkit; GQ = genotype quality; GoF = gain-of-function; ID = intellectual disability; LGS = Lennox–Gastaut Syndrome; LoF = loss-of-function; MAND = MBD5-associated neurodevelopmental disorder; NDD = neurodevelopmental disorder; OR = odds-ratio; p = protein change; PC = principal component; RP-PCR = repeat-primed PCR; SCA = spinocerebellar ataxia; SNP = single nucleotide polymorphism; SNV = single nucleotide variant; SUDEP = sudden unexpected death in epilepsy; TR = tandem repeat; TRF = Tandem Repeat Finder; UTR = untranslated region; VUS = variant of unknown significance; WGS = whole-genome sequencing; WES = whole-exome sequencing.

Graphical Abstract

A cohort of 30 adults with unexplained developmental and epileptic encephalopathies, for whom previous genetic tests were inconclusive.

Using whole genome sequencing, we identified pathogenic or likely pathogenic genetic variants in 32.1% (9/28) of the cohort, and two tandem repeat expansions:

- Single nucleotide variants (n=7)
- Copy number variants (n=2)
- Tandem repeat expansions (n=2)

Clinically relevant genetic variants impacting the genes marked with an * were identified in four of the six patients with Lennox Gastaut Syndrome.
Introduction

Epilepsies are a group of common neurological disorders which are primarily characterized by their enduring predisposition to generate recurrent seizures.1–3 Genetic factors contribute to the aetiology of epilepsy, where to date, over 900 epilepsy-associated genes have been identified.1–7 Interestingly, only a small proportion of known epilepsy-associated genes encode for ligand (10%) and voltage-gated (17%) ion channels.4–7 New genetic mechanisms continue to be implicated in both focal and generalized epilepsies.3,8–12 For example, Lennox–Gastaut Syndrome (LGS) is a severe childhood-onset epilepsy syndrome that may be caused by acquired mechanisms (e.g. brain injuries and hypoxic ischaemic lesions), structural abnormalities and/or genetic causes.13,14 Despite this known genetic basis, the molecular diagnosis of epilepsies remains challenging, with current studies capturing only a portion of the contributing genetic variation.5,15 This can partially be attributed to the incomplete assessment of genetic variants by most current genetic analysis methods. For example, in addition to single nucleotide variation, in-tronic (TTTTA)n/(TTTCA)n tandem repeat (TR) expansions have recently been implicated in benign familial adult myoclonic epilepsy (BAFME), suggesting that TR expansions may be a more common mechanism in epilepsies than currently reported.16–19 Studies interrogating the full spectrum of genomic variants, especially those utilizing whole-genome sequencing (WGS), will provide insights into epilepsy genetics and help resolve epilepsies of unknown aetiology.

In this study, we used WGS to identify rare genetic variation in a cohort of adults with developmental epileptic encephalopathies (DEEs), for whom all prior genetic tests were negative or inconclusive.

Materials and methods

Cohort recruitment

Adults with DEEs (n = 30) were recruited from the Adult Epilepsy Genetics Program at Toronto Western Hospital. All patients had a prior negative or inconclusive genetic test. This study was approved by the University Health Network’s Research Ethics Board.

Whole-genome sequencing

Of the 30 patients, there were two pairs of identical twins. Parents were also sequenced where possible (n = 11 pairs). In brief, genomic DNA was extracted from whole blood, and then sequenced using the Illumina HiSeq X platform at The Centre for Applied Genomics. Sequence reads were aligned to the GRCh37 reference genome using the Burrows-Wheeler Aligner read alignment program.20 Local realignment, base quality recalibration and removal of duplicate reads were carried out using the Broad Institute’s Genome Analysis Toolkit (GATK) package. The following algorithms were used for variant calling and genotyping: (i) GATK Haplotype Caller for single nucleotide variants (SNVs) and indels; (ii) ERDS (v1.1) and CNVnator (v0.3.2) for copy number variants (CNVs); and (iii) a combination of ExpansionHunter Denovo (EHdn, v0.7), Tandem Repeat Finder (TRF) and ExpansionHunter (EH, v3.0.2) for TRs.21–23

Each variant call format file was annotated using a custom ANNOVAR pipeline.24 To filter for only high quality SNVs, the following parameters were applied: (i) autosomal heterozygous variants met a genotype quality (GQ) cut-off value of ≥99, and an alternative allele fraction ≥0.3 and ≤0.7; (ii) homozygous or chromosome X variants had a GQ ≥ 25 and an alternative allele fraction >0.7; and (iii) all variants passed GATK pipeline filter constraints. DeNovoGear was used to detect de novo SNVs in patients sequenced in trio (n = 13, where two of the families have twins).25 To filter for only robust CNVs, CNVs had to be called by both ERDS and CNVnator.23 For TRs, EHdn was used to estimate the size and location of TRs lengths in >60 known TR-associated disease loci, as previously described.23,26 Ancestry and kinship analyses were carried out using PLINK (v1.9), as previously described.27

Rare variant analysis

A rare variant was defined as being present with <1% frequency in the general population, using genomic databases (the 1000 Genomes project, Exome Aggregation Consortium, Genome Aggregation Database, DECIPHER and the Database of Genomic Variants). Rare SNVs and CNVs impacting both coding and non-coding regions of the genome were analysed, as previously described.27,28 TR expansions were filtered based on the genomic content disrupted, biological relevance, known disease associations, and any overlap with reported literature or clinical reports in databases. Using the American College of Medical Genetics and Genomics’ (ACMG) guidelines, we classified rare SNVs and CNVs into pathogenic (i.e. disease-causing), likely pathogenic, variant of unknown significance (VUS), benign and likely benign categories.29–32 If a VUS was identified in a gene without a validated association to the patient’s phenotype, it was considered a gene of uncertain significance.29 As there are no ACMG standards to guide the interpretation of the pathogenicity of TR expansions, we defined a repeat expansion to be of interest if its size either (i) fell into the reported disease-causing range, or (ii) was larger than what is observed in control individuals in reported studies.

Variant validation

Where possible, variants were experimentally validated using either (i) Sanger Sequencing for SNVs; (ii) qPCR for CNVs; or (iii) Southern blotting and/or repeat-primed
PCR (RP-PCR) for TR expansions. Specifically, southern blotting was used to validate the CGG expansion in DIP2B found in patient 4 as previously described. In brief, genomic DNA was first digested using selected restriction-endonuclease enzymes, followed by the resolution of DNA fragments on an agarose gel. These DNA fragments were then transferred to a nylon membrane, and then subjected to hybridization analysis, where radioactively labelled probes were used to detect the sizes of the CGG alleles in DIP2B.

To validate the CTG repeat expansion in ATXN8OS in both patient 6 and his mother (6A), a combination of primer sequences were used to amplify the CTG repeat locus in ATXN8OS: a single forward fluorescently labelled primer (P1, 5'-FAM-CTGGGTCTTCTCATGTT AGAAAAACCT-3'), and a combination of two reverse primers in a 1:10 ratio (P3, 5'-TACGCATCCCCAGTT TGAGACGC-3', and P4, 5'-TACGCATCCCCAGTT TGAGACGCAGACGACGACGACGCA-3'). The reverse primer P4 anneals at different sites along the CTG repeat, resulting in a range of PCR product sizes up to the inclusion of the full repeat, depending on the amplification limit of the assay. These fluorescently labelled PCR products of different lengths were analysed using capillary electrophoresis and visualized using PeakScanner 2.

Statistical analysis: comparison of clinical characteristics

One-sided 2×2 Fisher Exact Tests were carried out to compare clinical characteristics between patients with an identified variant, and those with no identified variant, including sex, age of seizure onset, EEG, MRI, presence/absence of family history, developmental regression (DR) and intellectual disability (ID) severity. In each case, an odds-ratio (OR) and a P-value (P) were generated.

Data availability

The dataset(s) supporting the conclusions of this article is(are) included within the manuscript. Individual ancestries and kinship values are not included in order to preserve participants’ privacy.

Results

The cohort had 30 patients in total (19 males), all with DEEs. Six patients were diagnosed with LGS. The median age of seizure onset was 3 years (range: 17 days to 17 years old). Common clinical characteristics included epileptiform interictal findings in their paediatric or adult EEGs (n = 24, 80%), ID (n = 30, 100%), autism spectrum disorder (ASD, n = 10, 33.3%), and a previous family history of epilepsy or a neurodevelopmental disorder (NDD) (n = 19, 63.3%). Seven patients had psychiatric comorbidities, such as ADHD, anxiety and schizophrenia (n = 7, 23.3%). This cohort includes one adopted patient (Patient 19), one offspring of a consanguineous marriage (Patient 16) and two pairs of identical twins (Patients 8 & 12; Patients 29 & 30). All patients had previously undergone extensive investigation during their paediatric years. During their years in the Toronto Western Hospital’s Adult Epilepsy Genetics Program, the patients completed chromosomal microarrays (CMAs) (n = 25, 83.3%), large gene panels (n = 25, 83.3%) or whole-exome sequencing (WES) (n = 5, 16.7%).

WGS analysis

In total, 52 genomes were sequenced: 30 patients and 22 parents (Supplementary Table 1, Supplementary Fig. 1). There was an average read depth of 37.0 across the entire cohort. Overall, WGS detected an average of 4535 791 indels and SNVs, and 706 CNVs per genome amongst patients. Regarding rare variants, patients had an average of 307 indels and SNVs, and 4.1 CNVs impacting coding regions per genome.

Ancestry analyses found that the cohort was largely European (n = 40, 76.9%), with the remainder of patients belonging to South Asian (n = 9, 17.3%), American (n = 2, 3.85%) and East Asian (n = 1, 1.92%) ancestry groups (Supplementary Fig. 2). We confirmed that patient 16’s parents (16A, 16B) were third-degree relatives, and that the two twin pairs were genetically identical. Only one individual in each twin pair was included in downstream analyses, resulting in a total of 28 patients’ WGS data for analysis.

Clinically relevant rare variant analysis

Upon rare variant analysis, we identified seven pathogenic or likely pathogenic SNVs, and two pathogenic CNVs, resulting in a molecular diagnosis in 9/28 (32.1%) patients (Tables 1 and 2).

In addition, we identified two de novo VUS SNVs, which do not meet clinical significance as per ACMG guidelines, but instead are potentially clinically relevant and merit further investigation. We also identified two TR expansions in DIP2B and ATXN8OS. Overall, patients with an identified variant had a younger age of seizure onset compared to the variant absent group (OR: 8.27, P = 0.042, one-sided Fisher’s Exact Test) (Table 3).

Single nucleotide variants

Patient 24: We identified a novel rare pathogenic variant (p.Gln96fs) in NPRL3, of unknown inheritance, which results in a frameshift deletion. Heterozygous loss-of-function (LoF) variants in NPRL3 have been implicated in autosomal dominant focal epilepsies.
| Patient | Sex | Syndrome | Age of seizure onset (years) | Phenotype description |
|---------|-----|----------|-----------------------------|-----------------------|
| 24      | M   | DEE      | 17                          | A 34-year-old patient has epilepsy, with abnormal EEG (unclear if frontal or generalized), mild ID and ASD diagnosis. Patient’s mother, maternal aunt and uncle had seizures but were not available for testing. |
| 16      | F   | Early onset epileptic encephalopathy | 0.5 (6 months) | A 18-year-old patient has multifocal epilepsy, abnormal EEG, developmental delay (motor, speech, and language), severe ID and ASD diagnosis. Patient’s brother presented with seizures in early childhood but is seizure free. Mother has mild ID. |
| 29; 30* | M   | Early onset epileptic encephalopathy | 0.12 (6 weeks) | 34-year-old identical twins with an epilepsy of unclear localization, who presented with an abnormal EEG and normal MRI, developmental delay (motor, speech, and language), severe ID, dysmorphism and severe spasticity. Both were born prematurely. Patient 30 is blind. Patient 29 presented with DR. |
| 9       | M   | Early onset epileptic encephalopathy | 1.5            | A 30-year-old patient has myoclonic-atonic seizures, with an abnormal EEG, severe ID and severe ASD diagnosis (at age three years old). There is no family history of epilepsy. Patient’s sister also has an ASD diagnosis. |
| 19      | F   | Early onset epileptic encephalopathy | 0.67 (8 months) | Patient had a multifocal epilepsy, with an abnormal EEG and normal MRI, mild ID, depression and a mild ASD diagnosis. Patient passed away from SUDEP at 26 years old. Family history is unknown as the patient was adopted. |
| 3       | F   | Early onset epileptic encephalopathy | 1              | A 40-year-old patient has an epilepsy with seizure onset not localizable, with an abnormal EEG, severe ID, speech delay, dystonia and ataxia. Patient’s father’s first cousin had developmental delay and seizures. |
| 27      | F   | LGS      | 0.05 (17 days)              | A 17-year-old patient has a multifocal epilepsy, slow generalized spike and wave, generalized paroxysmal fast activity and MISF. MRI is normal. Developmental delay (motor, speech and language), hypotonia, DR, dysmetria and dysdiadokokinesia, severe ID and an ASD diagnosis. There is no family history of epilepsy. Patient’s brother has ADHD. |
| 10      | F   | LGS      | 3                           | A 23-year-old patient has multifocal and generalized seizures. EEGs show generalized paroxysmal fast activity, slow generalized spike, and wave discharges, MISF and generalized slow background activity. Patient has developmental delay (speech and language), later also had DR, moderate ID, short stature, weight and high below the 3rd percentile, and a movement disorder. She was diagnosed with diffuse muscle weakness and truncal instability, collapsing kyphosis, hypogonadism, and growth hormone deficiency. She was conceived through in vitro fertilization. Her twin sister died in uterus due to alobar anencephaly. This patient was a hypotonic baby. She sat at one-year-old, walked at two years, said her first words at four years, and was toilet trained at six years. The patient had moderate ID, and at the age of eight years, she started showing regression. She is completely non-verbal, inconsistently makes eye contact, is wheelchair bound, has lost sphincter control, and is fed through a G-tube. |
| 26      | F   | DEE      | 3                           | A 17-year-old patient has multifocal epilepsy with abnormal EEG and normal MRI, developmental delay (speech and language), and severe ID. Patient’s brother had two episodes of seizures at the age of nine years old. |
| 6       | M   | LGS      | 4                           | A 26-year-old patient has LGS with multifocal and generalized epilepsy, EEG showing slow generalized spike and wave, generalized paroxysmal fast activity and MISF. MRI is normal, mild ID, and a ASD diagnosis. There is no family history of epilepsy or ataxia. |

(continued)
Table 1 Continued

| Patient | Sex | Syndrome | Age of seizure onset (years) | Phenotype description |
|---------|-----|----------|----------------------------|----------------------|
| 4       | M   | LGS      | 1                          | Born at term after an uncomplicated pregnancy. A 20-year-old patient has LGS with multifocal and generalized epilepsy. EEG showing slow generalized spike and wave, generalized paroxysmal fast activity, MISF and slow background. Normal MRI, developmental delay (speech and language), later also had DR and moderate ID. Patient’s father has nocturnal seizures and has been treated with carbamazepine since the age of 14 years old. |

The * indicates that patients 29 and 30 are identical (i.e. monozygotic) twins. DR, developmental regression; LGS: Lennox–Gastaut syndrome; MISF, multiple independent spike foci; SUDEP, sudden unexpected death in epilepsy.

Table 2 Molecular findings identified by WGS in this cohort

| Patient | Variant type | Gene | Variant | Coordinate; Transcript | De Novo or Inherited | Interpretation; Evidence |
|---------|--------------|------|---------|------------------------|---------------------|--------------------------|
| 24      | SNV          | NPRL3| p.Gln96fs; c.287delA | Chr16:162,646; NM_001242247 | NA | Pathogenic: PVS1, PM2, PP4 |
| 16      | SNV          | KCNA1| p.Gln426Ter; c.1276T | Chr12:15,021,820; NM_000217 | Maternally inherited | Likely Pathogenic: PVS1 Moderate, PM2 |
| 29, 30* | SNV          | STXBP1| p.Arg292His; c.G875A | Chr9:130,430,439; NM_003165 | De novo | Likely Pathogenic: PS2, PM2, PP2, PP3, PP4 |
| 9       | SNV          | SLC6A1| c.Gly889Cys; p.G297R | Chr3:11,067,498; NM_003042 | NA | Pathogenic: PS1, PM2, PM5, PP2, PP3, PP4 |
| 19      | SNV          | KCNA2| p.Lys445fs; c.1334delA | Chr1:11,146,070; NM_004974 | NA | Pathogenic: PVS1, PM2, PP4 |
| 3       | SNV          | KCNA2| p.Arg297Gln; c.G890A | Chr1:11,146,515; NM_001204269 | De novo | Pathogenic: PS1, PS2, PM2, PP2, PP3, PP4 |
|         | SNV          | KCNIP4| p.Gln96Ter; c.C286T | Chr4:20,736,316; NM_001035004 | De novo | VUS: PS2, PM2 |
| 27      | SNV          | KCNA2| p.Glu183Lys; c.G547A | Chr1:11,146,858; NM_004974 | De novo | Likely Pathogenic: PS2, PM2, PP2, PP3, PP4 |
| 10      | CNV          | ORC4 | c.3925T | Chr12:120,867,801–123,012,800 | NA | Pathogenic: 1A, 2C-1, 3A, 4B, 5F |
| 26      | CNV          | 38 genes | DEL | Size: 2,145,000 | NA | Likely Pathogenic: 1A, 3A, 4C, 5F |
| 6       | TR           | ATXN8OS | 19 and ~135 CTG units | Chr13:70713516–70713651 | NA | NA |
| 4       | TR           | DIP2B | 12 and 128 CGG units | Chr12:50898877–50898807 | NA | NA |

All variants are described in relation to the coding DNA reference sequence (i.e. ‘c.’), the predicted consequences on the protein level (‘p.’), and transcript (‘NM.’). Variant interpretation is according to the ACMG guidelines (see Methods).

The * indicates that patients 29 and 30 are identical (i.e. monozygotic) twins. bp, base pair; Chr, chromosome; CNV, copy number variant; del/DEL, deletion; c, coding DNA; NA, not applicable; p, protein; SNV, single nucleotide variant; TR, tandem repeat; VUS, variant of uncertain significance.
Table 3 Comparison of clinical features between variant present/absent group

| Clinical feature           | Odds ratio | P-value |
|----------------------------|------------|---------|
| Sex                        | 2.77       | 0.175   |
| First seizure (Before five years old) | 8.27       | 0.042   |
| EEG findings               | ∞          | 0.175   |
| MRI findings               | 0.39       | 0.938   |
| Family history             | 1.44       | 0.493   |
| Developmental regression   | 1.05       | 0.632   |
| Intellectual disability    | 2.90       | 0.175   |
| ASD diagnosis              | 1.4        | 0.500   |
| Psychiatric comorbidities  | 0.0        | 0.033   |

Comparison of clinical features was done by Fisher’s Exact Test (one-sided). ASD, autism spectrum disorder.

Patient 16 harboured a maternally inherited rare likely pathogenic variant (p.Gln426Ter) in the C-terminus protein domain, which is found in the second exon of KCNA1. The patient’s mother is mildly affected.

Patient 29 harboured a de novo likely pathogenic missense variant (p.Arg292His) in STXBP1, which was also present in his monozygotic twin, patient 30. This is a novel change at an amino acid residue where pathogenic variants have been reported previously. The patients’ phenotype is compatible with STXBP1 encephalopathy.

Patient 9: We identified a rare pathogenic missense variant (p.Gly297Arg), of unknown inheritance, in SLC6A1. This variant has previously been reported in a 16-year-old with myoclonic adult epilepsy, and is in keeping with this patient’s phenotype.36

Three patients (19, 3 and 27) had three different pathogenic SNVs in KCNA2, but patients 3 and 27 also had de novo variants in other genes (KCNIP4 and UBR5) (Fig. 1). Clinically, all 3 patients with pathogenic KCNA2 variants had seizures, ID and ASD, but the patients with a second de novo variant also had movement disorders, as described below.

Patient 19 presented with seizure onset at three years of age, mild ID, and mild ASD. She passed away from sudden unexpected death in epilepsy (SUDEP) at 26 years old. She had a novel rare pathogenic variant in KCNA2 (p.Lys445fs).

Patient 3 presented with early onset seizure, severe ID and ASD, speech delay, dystonia, and ataxia. She had a rare pathogenic variant in KCNA2 (p.Arg297Gln), which has previously been reported. She had a second de novo nonsense variant (p.Gln96Ter) impacting KCNIP4. This was classified as a VUS: KCNIP4 is a gene of uncertain significance and is not associated with a disorder in humans, yet.

Patient 27 presented with seizure onset at 17 days old. She had infantile spasms, and later tonic, myoclonic, atypical absence and generalized tonic clonic seizures, evolving to a typical LGS phenotype. She also presented with severe ID, ASD, developmental delay (DD), DR, hypotonia, dysmetria and dysdiadochokinesia. Today, this patient is wheelchair bound, non-verbal, not toilet trained, and is fed through a G-tube. In addition to the rare likely pathogenic variant in KCNA2 (p.Glu183Lys), this patient also had a de novo missense variant (p.Pro1309Ser, VUS) in UBR5. This is also a gene of uncertain significance, as UBR5 is not associated with a disorder yet.

Copy number variants

Through WGS, we identified two clinically relevant CNVs. Patient 26 had a large likely pathogenic deletion (2 145 000 bp) on chromosome 12, spanning 38 genes.

Patient 10 is a female patient with LGS, who presented with afebrile seizure onset at three years of age (tonic, atonic, generalized tonic clonic and atypical absence). EEGs have shown fast paroxysmal activity, slow generalised sharp and wave discharges, multifocal independent spike foci and slow background. The patient’s seizures are drug resistant, but she has had a moderate temporary response to a ketogenic diet. Her height and weight are below the 3rd percentile, and her head circumference is below the 2nd percentile. Previous targeted MECP2 sequencing, metabolic investigations, an epilepsy gene panel and CMA (Affymetrix CytoScan HD SNP Array) were negative. Through WGS, a small pathogenic deletion (28 273 bp) was detected at the 2q23.1 locus, impacting both the coding and non-coding regions of MBDS (methyl-CpG-binding domain protein 5, OMIM 611472) and ORC4 (origin recognition complex subunit 4, OMIM 603056). Her phenotype is in keeping with the MBDS CNV.

TR expansions

We identified two TR expansions in two patients with LGS: a CGG repeat expansion in the 5′ untranslated region (UTR) of DIP2B in patient 4 (Fig. 2), and a CTG repeat expansion (of unknown inheritance) in ATXN8OS in patient 6 (Fig. 3). There are no ACMG guidelines to interpret TR pathogenicity yet: therefore, these two variants were not classified, but have potential clinical implications.

Patient 4: At the age of one year, he had several febrile generalized tonic clonic seizures. At 19 months, he developed infantile spasms, and later atypical absence, myoclonic, tonic, and focal onset seizures, and EEG findings in keeping with LGS. He has significant ID, is non-verbal and not toilet trained, but can ambulate and eat unassisted. He continues to present intractable seizures of multiple types. His father had previously presented with seizures (Fig. 2A). Through WGS, a 5′UTR CGG TR expansion was identified in the brain-expressed DIP2B (disco-interacting protein 2 homolog B) gene. This TR expansion has previously been reported in two individuals with ID and/or seizures.33

We confirmed the presence of an expanded allele in patient 4 using Southern blotting (Fig. 2B, Supplementary
Southern blotting estimated the allele sizes to be approximately 12 and 128 CGG repeat units in patient 4, which is larger than the initial estimates by our TR detection pipeline (12 and 99 units). Our TR detection pipeline estimated 12 and 15 CGG units in the mother, and 7 and 73 CGG units in the father.

Patient 6 has a history of seizures, with initial onset at four years of age. A brain MRI scan was normal. He underwent resective surgery, guided by cortical EEG with partial resections of the left frontal, anterior parietal and left mesial temporal lobes. Pathology identified a mild disorganization of neuronal lamination with clustering of neurons. Despite this extensive resection, this patient evolved with classic LGS, exhibiting typical clinical and electrophysiological markers. Later, he also had a partial corpus callosotomy, but still has drop seizures. He does...
not have ataxia. Despite upper limb ataxia, his brain MRI shows a normal cerebellum, without any evidence of volume loss. Through WGS, we identified a CTG expansion in \( \text{ATXN80S} \) specifically, our TR detection pipeline estimated 19 and 119 CTG repeat units in \( \text{ATXN80S} \). Using RP-PCR and capillary electrophoresis, we confirmed the presence of the expanded CTG repeats in \( \text{ATXN80S} \) in the proband: the repeat size is estimated to be greater than 135 CTG repeat units—the exact number cannot be determined as it is beyond the limit of this assay (Fig. 3B). We also verified the presence of a shorter expansion (~95 repeat units) in the mother (6A) (Fig. 3C). Previously, CTG TR expansions between 80 and 250 units in \( \text{ATXN80S} \) have been reported in spinocerebellar ataxia type 8 (SCA8).\(^{37}\)

**Discussion**

In this study, we used WGS to evaluate a cohort of adults with unexplained DEEs. Prior to WGS, our cohort completed a total of 55 molecular genetic tests. We identified a total of nine pathogenic or likely pathogenic variants in 28 patients, resulting in a 32.1% diagnostic yield (note: this excludes VUS, such as TR expansions and SNVs impacting genes of uncertain significance). Our study demonstrates that the examination of multiple types of rare genetic variation using WGS, including those found in the non-coding region of the genome, can help resolve unexplained epilepsies.

There are variable diagnostic rates seen across different genetic testing technologies. In particular, WGS provides a more uniform distribution of coverage depth, GQ, and minor read ratio, compared to WES.\(^{38-40}\) This is evident in diagnostic yields across various neurodevelopmental and neurological disorders—for example, the WES diagnostic rate in children with ASD is currently ~8.4%, but with the application of WGS, the diagnostic rate rises up to 11–25%.\(^{41-44}\) WGS also provides a complete coverage of the genome; in contrast, genes selected for analysis in commercial next-generation sequencing tests can vary significantly, potentially leading to the omission of causal genetic variants.\(^{38-40}\) Notably, a 100% WGS diagnostic yield has been reported in some small cohorts with specific epilepsy syndromes who previously had negative genetic tests, including a cohort of six individuals with severe-onset epilepsy, and 14 individuals with early infantile epileptic encephalopathies.\(^{45,46}\) In comparison, our interrogation of multiple types of genetic variants led to a diagnostic yield of 32.1%, but could have been different had we recruited from a more homogenous cohort of adults with unexplained DEEs. For example, a genetic cause has been identified in more than 90% of patients with Dravet syndrome, while only 65 to 75% of LGS cases have a clear identifiable cause (which can include
structural, metabolic or genetic causes).

All patients with LGS in this cohort (n = 6) had intractable epilepsy, including tonic seizures amongst other types, ID, and EEGs showing generalized slow spike and waves and generalized paroxysmal fast activity during sleep. WGS identified clinically relevant genetic variants in four of the six patients with LGS (66.6%). One LGS patient had a pathogenic variant in KCNA2, while a second had a pathogenic small CNV disrupting MBD5 and ORC4, which was not detected by an earlier CMA. This small deletion in the coding and non-coding regions of MBD5 would not have been identified with current CMA technology. Two LGS patients had two independent TR expansions with clinical implications (DIP2B and ATXN8OS)—this is the first ever such report of TR expansions in LGS.

**Novel TR expansions found in patients with LGS**

For patient 6, both a CMA and gene panel did not find a genetic cause for his LGS diagnosis. Through WGS, a trinucleotide CTG repeat expansion in ATXN8OS in the pathogenic range was identified. ATXN8OS repeat expansions are associated with SCA8: a progressive ataxia exhibiting reduced penetrance, where seizures are rare, but have been reported. Upon identifying the ATXN8OS repeat expansion, this patient was evaluated in the ataxia clinic to determine if the falls were due to ataxia. Despite upper limb ataxia, he does not have gait ataxia nor other hallmarks of SCA8. It is unclear if the dearth of SCA8 symptoms in patient 6 is caused by low penetrance, or if he will eventually develop other symptoms of SCA8 as he ages. However, it is also possible that repeat expansions in ATXN8OS may manifest as LGS. This would not be completely unexpected, as both SCA8 and some other forms of SCAs also manifest with seizures and ID. For example, Swaminathan reported a 22-year-old female patient with typical SCA8 who developed recurrent seizures and episodes of status epilepticus, which was successfully treated using antiepileptic therapies. In general, seizures and epilepsy are uncommon features in patients with SCAs: epilepsy as an inherent part of the phenotype has only been consistently described in SCA10 and SCA17, although an anecdotal association has also been reported in SCA2, SCA8, SCA13, SCA19/22 and SCA48.

We found that a second LGS patient, patient 4, harboured 12 and 128 CGG repeat units in DIP2B, compared to 6–23 units found in controls. This expansion has previously been reported to be associated with the fragile site FRA12A in two individuals with ID and/or seizures. The expansion in patient 4 exceeds the length of a normal allele by over 350 base pairs (>140 excess repeat units). Interestingly, DIP2B is highly expressed in the brain, mainly in the frontal and parietal cortices, making it a candidate gene for an epileptic disorder. The discovery of this CGG repeat expansion at 5’UTR of DIP2B in patient 4 adds to the limited cases in the literature, collectively suggesting that this expansion may contribute to ID and seizure-related phenotypes.

To date, TR expansions have been reported as a disease-causing mechanism in over 40 disorders, including spinocerebellar ataxias. To the best of our knowledge, repeat expansions have not previously been reported in LGS patients. TR expansions have been reported in patients with BAFMEs, which is associated with intronic (TTTTA)n(TTTC)n repeat expansions in SAMD12, YEATS2, STARD7 and MARCH6. We did not identify any such expansions in our cohort, suggesting that this may be a rare occurrence in patients with epilepsy, or is specific to BAFMEs. Interestingly, we investigated the genome-wide characteristics of TRs in 17 231 genomes of individuals with ASD, their families and control individuals, finding that TR expansions contribute a total of 2.6% risk to ASD. This suggests that TR expansions are not only responsible for movement disorders, and may be a more common mechanism in neurological disorders than currently reported.

**Small CNV in a patient with LGS**

We also identified a CNV that was too small (28 273 bp) to be detected by a CMA in patient 10 with LGS. Individuals with damaging genetic variants impacting MBD5 are collectively referred to as MBD5-associated neurodevelopmental disorders (MANDs). They exhibit DD and/or regression, ID, seizures, ASD-like behaviours, speech impairment, motor delays and hypotonia, which corresponds to patient 10’s phenotype. In addition, ORC4 is implicated in the autosomal recessive Meier-Gorlin syndrome, involving short stature and microcephaly. While patient 10 does not harbour a second variant impacting ORC4, her height and head circumference are lower than expected, suggesting that the ORC4 disruption may be modifying the patient’s overall core phenotype (which is due to MBD5 haploinsufficiency).

**Pathogenic variation in KCNA2 associated with abnormal cerebellar findings**

We identified three patients with pathogenic or likely pathogenic variants in KCNA2. KCNA2 encodes a voltage-gated delayed rectifier potassium channel, and is associated with various DEEs. Using either targeted gene sequencing or WES, Masnada et al. identified variants in KCNA2 in a cohort of 23 patients with epileptic encephalopathies, and found abnormal cerebellar findings in patients with KCNA2 with gain-of-function (GoF) pathogenic variants.

In our cohort, out of the three carriers of variants in KCNA2, two had significant cerebellar signs (Patients 27
and 3). Patient 27 also harboured a VUS in UBR5 (a ubiquitin-protein ligase component), which has previously been reported as potentially related to epilepsy, ASD and ID.\(^6,57–59\) Previously, a missense p. Arg1907His variant was reported in a family with BAFME, which also features cerebellar dysfunction.\(^57\) Since not all patients in the Masnada et al., 2017 cohort had undergone WES, it may be possible that some may have a variant impacting a second gene affecting cerebellar function, including those with GoF pathogenic variants in KCNA2. An alternative explanation is that the pathogenic variant in KCNA2 in our patient may be the only one responsible for patient 27’s phenotype, and that UBR5 remains a gene of uncertain significance.

Patient 3 had seizures, ID, dystonia and ataxia. In addition to the GoF p. R297Q variant in KCNA2 (which might explain the ataxia and dystonia), she also had a de novo VUS in KCNIP4.\(^29,55,56\) KCNIP4 encodes a potassium channel-interacting protein expressed in all regions of the brain, and is involved in regulating the frequency of slow repetitive firing and back-propagation of action potentials.\(^60,61\) It has been suggested as a candidate gene for ADHD, epilepsy and personality disorders, though there is only a single case study and one genome-wide association study to support this.\(^60,61\) Jenkins et al., 2022 recently reported that the canine KCNIP4 is implicated in two Norwegian Buhund dogs with progressive cerebellar ataxia, suggesting that it may be a potential candidate for cerebellar ataxia in humans and other species. Taken altogether, we propose that these additional de novo variants in KCNIP4 and UBR5 genes may contribute to the more severe ID and movement disorders seen only in patients 3 and 27, but not on the other KCNA2 variant carrier, patient 19 (who presented with epilepsy, mild ID, mild ASD and passed away from SUDEP). Sequencing KCNIP4 and UBR5 in larger, well-characterized cohorts will be necessary to determine their association with epilepsy, ID, and movement disorders.

**Limitations**

While we achieved a 32.1% diagnostic yield, it is important to note that our patients were previously screened by various next generation sequencing tests, and the overall yield would likely have been higher if there was an unbiased patient recruitment. It is also important to note that the nine rare genetic variants we identified are either disease-causing (pathogenic) or have a high likelihood to be disease-causing (likely pathogenic) for epilepsy, but may not be the sole variants contributing to the patient’s phenotype (i.e. there may be other variants contributing to the patient’s phenotype either independently or collectively). We did not evaluate variants with lower effect sizes (e.g. common variants) as this is a small cohort. We also did not analyse somatic variation present in resected tissues. This approach has previously identified a ‘two-hit’ phenomenon, where a combination of a germline and a second somatic variant can lead to focal epilepsies.\(^8\) Finally, the two reported TR expansions cannot be classified as there are no guidelines to facilitate pathogenicity interpretation, although these expansions are in the disease range size for other diseases.\(^53\)

**Conclusion**

WGS increased the diagnostic yield in our patients, previously studied with next-generation sequencing technology, by 32.1%. A majority of the identified variants (excluding those found in non-coding regions, and TR expansions) should have been detected by early next-generation sequencing tests, but were likely missed due to variability in commercial genetic testing over the years. This points to the utility of WGS in helping to resolve previously unexplained epilepsies. This is also the first report of TR expansions identified in patients with LGS, which will benefit from further functional characterization to establish causality.

**Supplementary material**

Supplementary material is available at Brain Communications online.

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**Competing interests**

The authors report no competing interests.

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