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ORIGINAL ARTICLE

Confirming the contribution and genetic spectrum of de novo mutation in infantile spasms: Evidence from a Chinese cohort

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Abstract

Objective: We determined the yield, genetic spectrum, and actual origin of de novo mutations (DNMs) for infantile spasms (ISs) in a Chinese cohort. The efficacy of levetiracetam (LEV) for *STXBP1*-related ISs was explored also.

Methods: Targeted sequencing of 153 epilepsy-related candidate genes was applied to 289 Chinese patients with undiagnosed ISs. Trio-based amplicon deep sequencing was used for all DNMs to distinguish somatic/mosaic mutations from germline ones.

Results: Total of 26 DNMs were identified from 289 recruited Chinese patients with undiagnosed ISs. Among them, 24 DNMs were interpreted as pathogenic mutations based on American College of Medical Genetics and Genomics guidelines, contributing to 8.3% (24/289) of diagnosis yield in the Chinese IS cohort. *CDKL5* and *STXBP1* are the top genes with recurrent DNMs, accounting for 3.1% (9/289) of yield. Further deep resequencing for the trio members showed that 22.7% (5/22) of DNMs are actually somatic in the proband or a parent. These somatic carriers presented milder seizure attacks than those with true germline DNMs. After treatment with LEV for half a year, three patients with DNM in *STXBP1* showed improved clinical symptoms, including seizure-free and normal electroencephalogram, except for a patient with a second DNM in *DIAPH3*.

Liying Liu and Fang Liu contributed equally to this work.

Significance: Our study confirmed the contribution and genetic spectrum of DNMs in Chinese IS patients. Somatic mutation account for a quarter of DNMs in IS cases. Treatment with LEV improved the prognosis of _STXBP1_-related ISs.

KEYWORDS

de novo mutation, infantile spasms, levetiracetam, somatic or mosaicism mutation, _STXBP1_

1 | INTRODUCTION

Infantile spasms (ISs) are a common form of epileptic encephalopathy (EE) in infancy, with an incidence of 2–5/10,000 live births (Lux & Osborne, 2004). Hypsarrhythmia in the electroencephalogram (EEG) is the key diagnosable sign of ISs, which usually occur in clusters. For infants and young children with ISs, the neurodevelopmental delay is the primary concern of both neurologists and parents, as it has been shown that the majority of patients (85%) with ISs have developmental delay (Yuskaitis et al., 2018). However, long-term follow-up has also shown that improved neurodevelopmental outcomes can be achieved in up to 23.6% of IS patients with early diagnosis and intervention (Widjaja et al., 2014), indicating that early diagnosis improves the neurodevelopmental status of ISs.

As a subtype of EE, ISs have a high degree of clinical and genotypic heterogeneity. Clinically, ISs show highly dynamic evolution between different age groups. For example, Ohtahara syndrome evolves to West syndrome or to Lennox–Gastaut syndrome as the age of onset changes (McTague et al., 2015). Genetically, approximately 70 genes including 43 dominant and 22 recessive genes involving ion channels, synaptic regulation, and developmental functions, have been identified as definitive causative genes of human EE (Carvill et al., 2013; EuroEPINOMICS-RES Consortium et al., 2014; Lindy et al., 2018; McTague et al., 2015). With rapid advances in high-throughput genomic sequencing techniques, the diagnostic yield for monogenic mutations has increased 10%–50%, depending on the number of genes assayed, case inclusion, and age of onset (Allen et al., 2013; Carvill et al., 2013; Helbig et al., 2016; Lemke et al., 2012; Lindy et al., 2018; McTague et al., 2015; Trump et al., 2016). However, each gene is individually responsible for less than 1% of EE cases (Noebels, 2015; Ottman et al., 2010), implying complex genotypic heterogeneity of ISs. Besides the germline mutations, somatic mosaicism has recently emerged as an important cause of EE or ISs (Depienne et al., 2010; Jdila et al., 2017; Kato et al., 2015; Masliah-Plachon et al., 2010; Milh et al., 2015; Saitsu et al., 2010).

Another important advance in our understanding of the genetic basis of ISs is the identification of de novo mutations (DNMs). These include both copy number variants (CNVs) and single-nucleotide variants (SNVs), which have been recognized as important causes of childhood EE or ISs (Allen et al., 2013; Claes et al., 2001; Epi4K Consortium, 2016; EuroEPINOMICS-RES Consortium et al., 2014; Hamdan et al., 2017; Saitsu et al., 2008), also explaining the sporadic occurrence of ISs. Chromosomal microarray analyses performed in patients with ISs have shown that up to 6.8% of IS cases carry de novo pathogenic or likely pathogenic CNVs (Mefford et al., 2011). In 2013, the Epi4K Consortium performed the first trio-based whole-exome sequencing (WES) study for 264 families affected by ISs or Lennox–Gastaut syndrome. Their data showed that 7% of analyzed patients had a definitive causal DNM (Allen et al., 2013). Their further study on 356 trio-based WES cases demonstrated a 12% yield (EuroEPINOMICS-RES Consortium et al., 2014). Another cohort study from 8565 Western patients with both epilepsy and neurodevelopmental disorders tested via 70-gene targeted sequencing showed that 72% of 417 deleterious variants were DNMs in dominant genes (Lindy et al., 2018).

The monogenic diagnostic yield of Chinese patients with EE has been reported (Miao et al., 2018; Yang et al., 2018; Zhang et al., 2016). The yields of causative mutations varied between 18% and 42% depending on the age of onset, sample, and panel size. However, DNM was reported only in very small cohorts with single gene assayed (Xie et al., 2019), or sporadically for individual case presentations (Li et al., 2018). Therefore, the actual contribution of DNMs in Chinese IS patients are still not well clarified. Studying the contribution and genetic spectrum of DNMs to ISs can facilitate the genetic diagnostic strategy, leading to patients with a negative family history of EE being referred to genetic testing.

In this study, we applied the targeted sequencing to cover the coding regions of 153 epilepsy-related genes, quickly screening the pathogenic DNMs across a Chinese IS cohort. In addition, we used trio-based deep sequencing to confirm the origin of the DNMs, distinguishing somatic/mosaic mutations from germline ones. Finally, we explored the efficacy of levetiracetam (LEV) therapy for _STXBP1_-related ISs.

2 | MATERIALS AND METHODS

2.1 | Patient recruitment

Children with ISs referred to Children's Medical Center of the Chinese People's Liberation Army General Hospital were

recruited from June 2015 to December 2017. All patients fulfilled the diagnostic criteria of the West Delphi Group (Lux & Osborne, 2004). The clinical manifestations, EEG, and brain MRI of each recruited patient were collected. All participants completed the routine genetic screening procedure (including karyotyping, *TSC1/TSC2* sequencing and MLPA, metabolic disease screening). Only patient with negative results were recruited.

2.2 | Target panel sequencing and variant calling

Genomic DNA was extracted from peripheral blood leukocytes of all probands and their parents using a DNA extraction kit (TIANGEN, Beijing, China). The target genes included the published gene list of the Epi4K Consortium (EuroEPINOMICS-RES Consortium et al., 2014) and the curated genes in six databases (OMIM, GeneCard, MelaCard, Orphanet, HPO, and HGMD) with the following query term: "epilepsy/seizure/IS". A total of 153 genes including 45 AR genes, 64 AD genes, 18 X-linked genes, and 26 candidate risk genes were chosen for sequencing (see Table S1). Target libraries were enriched by the GenCap custom enrichment kit (MyGenostics, Beijing, China) and were sequenced on an Illumina HiSeq2000 (Illumina, San Diego, USA) for paired-end reads of 150 bp. Quality controls were performed for raw data and the clean reads were mapped to the UCSC hg19 human reference genome using BWA. The SNP and indel variants were detected by GATK Haplotype Caller. Following this, SNVs with minor allele frequency (MAF) >0.1% or >1% (for dominant and recessive genes, respectively) reported in our in-house database, the dbSNP database, 1000 Genomes dataset, or gnomAD database were not recognized as rare SNVs. The functional effects of missense mutations were predicted by four algorithms (PolyPhen, Sorting Intolerant from Tolerant, Protein Analysis Through Evolutionary Relationships, and Pathogenic Mutation Prediction). Three disease-related databases (ClinVar, HGMD, COSMIC) were used to assess whether it is novel or reported pathogenic SNV.

The CNVkit tool of NextGENe software (version 2.4.1.2; SoftGenetics, PA. USA) was used to analyze and visualize the candidate CNVs. Only CNVs spanning more than five amplicons were chosen for validation using quantitative PCR (7500 Fast Real-Time PCR System, Thermo Fisher Scientific, see Table S2 for designed primers) or Agilent 4 × 180 K array-CGH chip (Agilent Technologies, CA, USA) (Figure S1).

2.3 | Mutation validation and family segregation analysis

All candidate SNVs detected from each proband were re-sequenced in their available core family members on an ABI

PRISM 3730 genetic analyzer (Thermo Fisher Scientific, MA, USA) after touchdown PCR. In order to avoid PCR dropout, we used two sets of primer pairs to validate each DNM. The segregation status of candidate variants was then used as the inheritance evidence during the process of interpreting the pathogenicity. We strictly followed the guidelines of the American College of Medical Genetics and Genomics (ACMG) to interpret the pathogenicity of SNVs (Richards et al., 2015) and CNVs (Riggs et al., 2019).

2.4 | Amplicon-based deep sequencing (ADS) for DNM

Amplicon-based deep sequencing (ADS) was used to detect somatic mutations, as described previously (Jiang et al., 2017). Genomic DNA from all 21 trios with 22 DNMs was amplified to generate the DNM-specific amplicon (Table S3). Only the primer pair which have been validated without amplification bias in Sanger sequencing was used. Using Ion-Plus Fragment Library Kit (Thermo Fisher Scientific), a sequencing library was prepared, purified, quantified, and enriched in Ion Sphere Particles (ISPs). Finally, enriched ISPs were loaded onto the 316 V2 chip and sequenced in an Ion Torrent Personal Genome Machine (Thermo Fisher Scientific, MA USA). Sequencing data from PGM were analyzed through the Integrative Genomics Viewer Version 2.3.25. The variant ratio was defined using the proportion of variant reads relative to the total sequencing reads.

Using the unpublished data of alleles with inherited germline heterozygous variants, we established the normal distribution curve of the variant ratio for heterozygous germline SNVs at appropriate sequencing coverage. With the mean and standard deviation (SD) of these normally distributed heterozygous germline SNVs, any DNM whose variant ratio lower than 2SD of Mean was automatically recognized as somatic mutation.

2.5 | Protein–protein interaction network analyses

We uploaded the gene list with de novo SNVs combined with the genes covered in the de novo CNVs to the online Ingenuity Pathway Analysis platform (IPA; Qiagen, CA, USA) to identify potential networks, related disorders, and canonical pathways.

2.6 | Treatment evaluation of LEV

Four patients with *STXBP1* DNMs were included to evaluate the efficacy of LEV. Their treatment history, category of

routine anti-epilepsy drugs (AEDs), the duration and dosage of LEV, the frequency of seizure attack, and EEG during treatment were recorded. We definite "effective to levetiracetam" when patient shows decreased seizure attack (≥50%). LEV was prescribed from 10 to 20 mg/kg/day (initial dosage) and increased gradually to 50 mg/kg/day (maximum dosage). The time of follow-up was from 7.3 to 19.77 months.

2.7 | Statistical analysis

Chi-squared/Fisher's exact test or Kruskal–Wallis test was used to compare the qualitative variables between two groups, and independent t-test was used to compare the quantitative variables between two groups. $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | General information on the Chinese IS cohort

A total of 289 undiagnosed patients were recruited for this study based on primary routine examinations (Table 1). The ratio of males to females was 1.39:1. The median age of enrolled children was 8.7 months (range: 0.07–36.8 months). The median seizure frequency was 35 times/day (0–905 times/day) and the first attack occurred at a median age of 4 months (range: 0.16–15 months). In addition, 18.7% of patients developed other seizure types in the progression of their disease, including focal seizures, tonic seizures, generalized tonic–clonic seizures, atonic seizures, and/or myoclonic seizures. Brain magnetic resonance imaging (MRI) was normal in 220 patients (76.1%). And 49 patients (17.0%) revealed nonspecific changes including nonfocal cortical and subcortical atrophy, delayed myelination, thinning of the corpus callosum, and/or T2 hyperintensity of basal ganglia.

3.2 | The diagnostic yield and DNM spectrum of the Chinese IS cohort

The mean coverage of the targeted sequencing was 250× and the region with >20× constituted 96% of the total. Null variants (nonsense, frameshift, canonical splice sites, initiation codon, single exon, or multiexon deletion) and deleterious missense variants predicted by at least two software programs were preferentially selected for Sanger validation and family segregation analysis. With this strategy, a total of 889 candidate SNVs and 4 CNVs were chosen for Sanger sequencing, real-time PCR, or aCGH. Paternity testing was performed for all probands with DNM using 12 STR alleles.

TABLE 1 General information on the Chinese IS cohort

General information	Number (%)
Sex	
Male	168 (58.1%)
Female	121 (41.9%)
Age at admission (months)	
Median	8.7
Range	0.07–36.8
Age at first attack (months)	
Median	4
Range	0.16–15
≥ two seizure type (%)	54 (18.7%)
Seizure frequency (times/day)	
Median	35
Range	0–905
MRI	
Normal	220 (76.1%)
Other changes	49 (17.0%)
NA	20 (6.9%)
Number of Antiepileptic drugs (AED)	
0 or NA	1 (0.3%)
1	87 (30.1%)
2	84 (29.1%)
3	75 (26.0%)
≥4	42 (14.5%)
Median	2
Range	0–8
Effects on discharged AED	
Seizure free	157 (54.3%)
Effective (≥50% attack)	62 (21.5%)
Invalid (<50% attack)	46 (15.9%)
NA	24 (8.3%)

Abbreviation: NA, not available.

In this Chinese IS cohort, six patients carried homozygous or complex *trans* heterozygous mutations in recessive genes, and 28 patients carried 29 DNMs in epilepsy-related genes, including 25 de novo SNVs (Patient 29 carried two DNMs: one in *DIAPH3* and the other in *STXBP1*) and 4 de novo CNVs. Further PCR experiments with the second set primer validated one false de novo SNV due to PCR dropout, and biological parental testing validated a non-parental relationship for another two de novo SNVs, leaving 22 SNVs and 4 CNVs in 25 patients. 24 DNMs except for *DIAPH3* and *CACNA1A* were evaluated as pathogenic or likely pathogenic in accordance with the ACMG guidelines (Richards et al., 2015; Riggs et al., 2019). In contrast, no autosomal recessive

TABLE 2 Detailed clinical information and genetic characteristics of patients with de novo SNVs detected in the Chinese IS cohort

ID/age gender	Onset age of seizure and type	Onset age of spasms	NDD comorbid[a]	EEG	Gene	Mutation (GRCh37/hg 19)	Zygosity	Reported or Novel	Classification
30/9 m/M	7 m IS	7 m	DD, ASD	Hypsarrhythmia	ARX NM_139058	chrX:25023007 G>A; c.1469C>T; p.Pro490Leu	Hemi.	Novel	Likely pathogenic (PS2+PM2+PP3)
44/6 m/M	4 m IS	4 m	DD, ASD	Multifocal	ARX NM_139058	chrX:25025525 C>T; c.1151G>A; p.Arg384His	Hemi.	Reported, conflicting interpretations	Likely pathogenic (PS2+PM2+PP3)
24/6 m/F	3 m IS	3 m	ASD	Hypsarrhythmia	CACNA1A NM_001127222	chr19:13318443 G>T; c.7205C>A; p.Pro2402Gln	Het.	Novel	VOUS (PS2+PM2)
2/8 m/F	0.25 m Focal	7 m	DD, ASD	Multifocal	CDKL5 NM_003159	chrX:18528955T>C; c.80T>C; p.Val27Ala	Het.	Novel	Likely pathogenic (PM1+PS2+PM2+PP3)
50/24 m/M	14 m IS	14 m	DD, ASD	Multifocal	CDKL5 NM_003159	chrX:18528976 C>A; c.99+2C>A; splicing site	Hemi.	Novel	Pathogenic (PVS1+PS2+PM2)
36/8 m/F	8 m IS	8 m	DD, ASD	Hypsarrhythmia	CDKL5 NM_003159	chrX:18598089 G>T; c.403+1G>T; splicing site	Het.	Reported as pathogenic, different nucleotide in same location	Pathogenic (PVS1+PS2+PM5+PM2)
39/5 m/M	2.5 m Tonic	4 m	DD, ASD	Multifocal	CDKL5 NM_003159	chrX:18622719 C>T; c.1675C>T; p.Arg559X	Hemi.	Reported as pathogenic	Pathogenic (PVS1+PS2+PS4_moderate+PM2)
34/17 m/F	0.7 m Focal	4 m	DD, ASD	Hypsarrhythmia	CDKL5 NM_003159	chrX:18622774-18622776, del GG; c.1731_1732del GG; p.Met577fs	Het.	Novel	Pathogenic (PVS1+PS2+PM2)
29/12 m/F	0.16 m Focal	5 m	ASD	Hypsarrhythmia	DIAPH3 NM_001042517	chr13:60565358 C>T; c.1295G>A; p.Arg432Lys	Het.	Novel	VUS (PM2+PS2_moderate)
47/13 m/F	4 m IS and Tonic	4 m	DD, ASD	Multifocal	GRIN2B NM_000834	chr12:13761698 A>T; c.1849T>A; p. Ser617Thr	Het.	Novel	Likely pathogenic (PS2+PM2+PP3)
46/15 m/M	4 m IS	4 m	DD, ASD	Hypsarrhythmia	KCNB1 NM_004975	chr20:47990350 G>A;.;2 c.1747C>T; p. Arg583*	Het.	Reported as Pathogenic	Pathogenic (PVS1+PS2+PS4_supporting+PM2)

(Continues)

TABLE 2 (Continued)

ID/age gender	Onset age of seizure and type	Onset age of spasms	NDD comorbid[a]	EEG	Gene	Mutation (GRCh37/hg 19)	Zygosity	Reported or Novel	Classification
32/17 m/F	14 m IS	14 m	DD, ASD	Hypsarrhythmia	KCNB1 NM_004975	chr20:47991468 G>A; c.629C>T; p.Thr210Met	Het.	Reported as Pathogenic	Likely pathogenic (PS2+PS4_supporting+PM2+PP3)
33/13 m/F	6 m IS	6 m	DD, ASD	Hypsarrhythmia	KCNQ2 NM_004518	chr20:62076109 C>T; c.593G>A; p.Arg198Gln	Het.	Reported, conflicting interpretations	Likely pathogenic (PS2+PM2+PS4_moderate+PP3)
42/23 m/F	6 m Tonic	13 m	DD, ASD	Multifocal	MEF2C NM_001131005	chr5:88119562 C>G; c.44G>C; p.Arg15Pro	Het.	Reported as pathogenic in same amino acid	Likely Pathogenic (PM5+PS2+PM2+PP3)
37/2 m/F	1 m IS	1 m	DD	Hypsarrhythmia	SCN2A NM_001040142	chr2:16624520 2 G>A; c.4886G>A; p.Arg1629His	Het.	Reported, conflicting interpretations	Likely pathogenic (PS2+PM2+PP3)
45/13 m/M	11 m IS	11 m	NA	Multifocal	SCN2A NM_001040142	chr2:166245511-166245512 delC; c.5196delC; p.Pro1733Lfs*36	Het.	Novel	Pathogenic (PVS1+PS2+PM2)
38/6 m/F	6.3 m IS	6.3 m	DD, ASD	Hypsarrhythmia	SCN8A NM_014191	chr12:52082568 G>A; c.641G>A; p.Gly214D	Het.	Reported as pathogenic	Likely pathogenic (PS2+PS4_supporting+PM2+PP3)
51/4 m/M	2.5 m IS	2.5 m	DD	Hypsarrhythmia	STXBP1 NM_003165	chr9:130420701 G>C; c.217G>C; p.Ala73Pro	Het.	Novel	Likely pathogenic (PS2+PM2+PP3)
31/3 m/F	2.5 m Focal	2.5 m	DD	Hypsarrhythmia	STXBP1 NM_003165	chr9:130438177-130438178 insG; c.1205_1206insG; p.Tyr402_D403delinsX	Het.	Novel	Pathogenic (PVS1+PS2+PM2)
35/3 m/F	1.7 m IS	1.7 m	DD, ASD	Hypsarrhythmia	STXBP1 NM_003165	chr9:130438970-130438970 delC; c.1297delC; p.Pro433fs	Het.	Novel	Pathogenic (PVS1+PS2+PM2)
29/12 m/F	0.16 m Focal	5 m	ASD	Hypsarrhythmia	STXBP1 NM_003165	chr9:130440783 G>A; c.1433G>A; p.Trp478X	Het.	Novel	Pathogenic (PVS1+PS2+PM2)
49/3 m/F	1 m IS	1 m	DD, ASD	Multifocal	TCF4 NM_001083962	chr18:53018122 A>G; c.482T>C; p.Leu161Pro NM_001083962NM_001083962	Het.	Novel	Likely pathogenic (PS2+PM2+PP3)

Abbreviation: NA, not available.

[a]Reported: have reported in the public disease-related databases including HGMD/ClinVar/ClinGen database.

variants were evaluated as pathogenic or likely pathogenic. Thus, the yield of pathogenic DNM from this undiagnosed Chinese IS cohort was 8.3% (24/289), indicating that DNMs of dominant genes rather than biallelic mutations of recessive genes contribute to the ISs in this Chinese cohort. The clinical and genetic information of these IS patients with DNMs were presented in Tables 2, 3 and S4. The validation and parental inheritance of de novo CNVs were described in Figure S1.

The DNMs spectrum comprised nine null SNVs, 13 deleterious missense SNVs on 12 genes. The genes with recurrent DNMs included *CDKL5* (*n* = 5), *STXBP1* (*n* = 4), *SCN2A* (*n* = 2), *ARX* (*n* = 2), and *KCNB1* (*n* = 2). DNMs of *CDKL5* and *STXBP1* account for 3.1% of the IS cohort. After reviewing the ClinVar and HGMD professional database, we confirmed nine recurrent pathogenic SNVs at specific nuclide acid sites as well as two recurrent CNVs which have previously been reported as pathogenic CNVs in Western and Chinese early-onset EE cohorts (Li et al., 2019; Michaud et al., 2014). In total, recurrent DNMs accounted for 46% (11/24) of the causative variants in the Chinese IS cohort. Among them, 1p36.33 deletion and 15q11.2–13.1 triplication are known syndromic genomic disorders. For 16p13.11 duplication in patient 72, although little evidence of triplosensitivity (TS=1) was reported on the ClinGen website, one recent large case-control study has proved 16p13.1 duplication is significantly associated with series of neurodevelopmental disorders with incomplete penetrance and variable expressivity (Allach El Khattabi et al., 2018).

Recruited patients were divided into two groups based on whether DNM was identified. Neither the sex (10/25 vs. 158/264, *p*=0.055) nor onset age (4.42 vs. 4.89, *p*=0.564) was significantly different in two groups. Although patients with DNM showed other seizure more often (36.0% vs. 17.0%, *p*=0.03), and showed more seizure frequency (128 vs. 69, *p*=0.192) than patient without DNM, the difference did not reach significance. The efficacy of routine AEDs in two groups was not significantly different also (48.0% vs. 60.4%, *p*=0.572).

The Gene Ontology functional analysis showed DNMs significant enriches in neurotransmission or the morphogenesis of neurons (Table 4). There are 13 de novo SNVs involve in this pathway, and seven de novo SNVs involved in ion channel. A close but direct connection was seen between genes affected by DNMs (Figure 1). Moreover, we found that 21/26 DNMs occurred in strongly conserved genes that are intolerant of loss-of-function (LOF) mutations (Table 4, pLI > 0.9), implying that, for these genes with DNMs, possibly it is haploinsufficiency via LOF rather than gain of function (GOF) contributes to ISs. In addition, we compared the enriched pathway of DNM genes with that of remained genes without DNM, no differential enriched pathway was seen in DNM genes.

TABLE 3 Detailed clinical information and genetic characteristics of patients with de novo CNVs detected in the Chinese IS cohort

ID/age gender	Onset age of seizure and type	Onset age of spasms	NDD 4	EEG	Cytogenic band (GRCh37/hg19)	Type	Number of involved gene	Reported or novel[a]	Classification
103/15 m/M	2 m Focal	3 m	DD	Multifocal	1p36.33-p36.32 (752721-3645052) x1	Deletion	82 genes including *GABRD*	1p36 deletion syndrome	Pathogenic
202/8.9 m/M	4 m IS	4 m	DD	NA	7q21.11 (77645000-79083000) x1	Deletion	8 genes including *MAGI2*	Reported as Pathogenic in literature[b]	Likely pathogenic
201/7 m/M	3 m Focal	4 m	DD, ASD	Multifocal	15q11.1-q13.3 (20095481-32880457) x4	Triplication	240 genes including *UBE3A, MAGEL1, GABRB3, SNRPN*	PWS/AS region duplication syndrome	Pathogenic
72/10 m/F	2 m IS	2 m	DD, ASD	Multifocal	16p13.11 (14910205-16561151) x3	Duplication	32 genes including *MYH11*	16p13.11 recurrent duplication	Likely pathogenic

Abbreviation: NA, not available.

[a]Reported: have reported in the public disease-related databases including HGMD/ClinVar/ClinGen database.

[b]Marshall CR, Young EJ, Pani AM, Freckmann ML, et al. Infantile spasms is associated with deletion of the *MAGI2* gene on chromosome 7q11.23-q21.11. Am J Hum Genet. 2008 Jul;83(1):106–11.

The main clinical features of *CDKL5*-related encephalopathy include early-onset seizures (usually occurring within the first three months) and severe neurodevelopmental problems (Fehr, Wong, et al., 2016). Male carriers have much more severe neurodevelopmental impairment than female carriers (Fehr, Downs, et al., 2016). In addition, the clinical outcome was found to be directly associated with the location of the *CDKL5* mutation. Mutations in the N-terminal kinase domain (aa 13 to aa 297) were associated with more severe clinical symptoms than mutations in the C-terminal region (Fehr, Downs, et al., 2016; Fehr, Wong, et al., 2016). As *CDKL5* is the gene most commonly showing DNMs ($n = 5$) in our Chinese IS cohort, we compared the relation between domain of DNM location and clinical phenotypes. The ratio of males to females was 2:3. The median age at spasms onset was 7 months (range: 4–14 months) and the median frequency of seizures was 55 times/day (range: 6–905 times/day). All patients had developmental delay and ASD. Three patients (Patients 2, 36, and 50) were found to carry mutation in the N-terminal kinase domain. Surprisingly, one male patient (Patient 50, c.99+2C>A) did not have a more severe clinical phenotype despite carrying a germline DNM in the N-terminal kinase domain, and he did not present IS attach until 14 months of age and became seizure-free with one anti-epileptic drug (AED). We assumed that some appropriately spliced transcript was possibly produced from this mutant allele. In addition, another male patient (Patient 39, c.1675C>T) also exhibited a moderate clinical phenotype (we will discuss this case later when considering the somatic mutation status).

3.3 | Somatic and germline mosaicism status of four de novo SNVs

A previous deep sequencing study of DNMs proved that a 39% mutant ratio is a reasonable cut-off to deviate somatic mutation from germline heterozygous mutations (Acuna-Hidalgo et al., 2015). With ADS for our germline heterozygous mutations (range: 405–600×, mean: 497.7×), the variant cut-off of somatic mutations in our ASD platform was 44%. To minimize the false identification of somatic mutations, we chose 40% as the cut-off to definite the somatic mutations. The mutation with allelic ratio higher than 1% in tested parents was considered as germline mosaicism.

The mean resequencing coverage of 22 de novo SNVs was 4633× (329–18,484×, Table S5). We found that four presumed germline DNMs were actually mosaic mutations in the blood of the offspring (Table 5), including two *STXBP1* null DNMs (Patient 29 and Patient 35), one *CDKL5* null DNM (Patient 39), and one *KCNQ2* missense DNM (Patient 33). We also found that Patient 44 with a germline missense DNM in *ARX* inherited this as a consequence of low-level (only 2%) mosaicism present in his unaffected mother (Table 5). In total, the somatic or germline mosaic mutation explained 22.7% (5/22) of de novo SNVs in our Chinese IS patients with the remainder having true germline DNMs. We also compared the phenotype between germline and somatic mutations in our cohorts. Patient 39 (*CDKL5*, 65% mutation) presented with a lower seizure rate compared with patient 50 with germline DNM (20 vs. 55 times/day).

TABLE 4 Pathways involved and the intolerant score of DNMs detected in the Chinese IS cohort

Diseases or functions annotation	*p* value	Gene name	Sample count in this IS cohort
Neurotransmission	9.73E-07	*CACNA1A, GABRD, GRIN2B, KCNB1, KCNQ2, SCN2A, SCN8A, STXBP1*	13
Development of neurons	2.05E-04	*ARX, CACNA1A, CDKL5, GABRB3, MAGI2, MEF2C, NDN, UBE3A*	13
Presynaptic compartments	–	*STXBP1*	3
Post-synaptic compartments	–	*GRIN2B, GABRD, GABRB3*	2
Ion channel	–	*CACNA1A, KCNB1, SCN2A, SCN8A, KCNQ2*	7
Intolerant score in ExAC database		**Gene name**	**Sample count in this IS cohort**
pLI > 0.98		*GRIN2B, STXBP1, KCNB1, SCN2A, SCN8A, CACNA1A, KCNQ2, UBE3A, TCF4, CDKL5, GABRB3, MYH11*	20
0.98 ≥ pLI > 0.9		*GABRD*	1
0.9 > pLI > 0.75		*ARX. MAGI2, SNRPN*	4

Note: The default Fisher's exact test was used to calculate a *p* value that determined the probability of the genes being involved in each pathway.

Path Designer Network 1

FIGURE 1 Protein–protein interaction network of DNM genes identified from the Chinese IS cohort. Genes are represented as nodes and only solid nodes indicate DNM genes in our cohort. Edges indicate known interactions between proteins (solid lines for direct interactions, dashed lines for mean indirect interactions). The gene shapes are indicative of molecular class, as defined by IPA: enzyme, rhombus; ion channel, vertical rectangle; kinase, inverted triangle; ligand-dependent nuclear receptor, horizontal rectangle; phosphatase, triangle; transcription regulator, horizontal oval; transmembrane receptor, vertical oval; transporter, trapezoid; and unknown, circle

3.4 | Efficiency of LEV treatment in patients with DNM in *STXBP1*

Three patients carry *STXBP1* null mutations (Patient 29, 31 and 35) and one patient carry missense mutation (Patient 51), in which two patients (Patient 29 and 35) presented somatic mutations. The median onset age of spasms was 2.5 months (1.7–5 months) and the median number of epileptic spasms was 242.5 times/day (129–650 times/day). EEG consistently showed hypsarrhythmia. All patients had various degrees of developmental delay, although the brain MRI results were normal except for patient 29. These clinical phenotypes were similar to those in other Western or Chinese EE cohorts (Li et al., 2018; Stamberger et al., 2016). Patients with germline null mutation (Patient 31, c.1205-1206insG) did not present

a higher spasms rate (320 vs. 650 times/day) or earlier onset age (2.5 vs. 2.5 m) than one with germline missense mutation (Patient 51, c.217G>A). But patient with somatic mutation (Patient 35, c.1297delC, 39% mutation) did present a lower seizure rate than two patients with a germline mutation (Patient 31 and 51) (165 vs. 320 or 650 times/day). Patient 29 carried a second DNM in *DIAPH3*. Lesca Gaetan previously described partial deletion of *DIAPH3* in a patient with EE of the Landau–Kleffner and continuous spikes and waves (Lesca et al., 2012). The epilepsy of Patient 29 was not under control with the routine AEDs including topiramate, valproate, low-dose LEV and corticotropin plus MgSO4.

The remission effect of levetiracetam (LEV) to early-onset EE has been reported sporadically in a patient with *STXBP1* mutation (Li et al., 2018; Milh et al., 2011; Vatta

TABLE 5 Somatic or germline mosaicism of DNM SNVs identified in the Chinese IS cohort

Sample ID	Tested sample	Gene	Mutation (GRCh37/hg 19)	Total coverage[a,b]	Read of alternate allele (%)[a,b]
29	Blood	*STXBP1* (NM_003165)	chr9:130440783 G>A; c.1433G>A; p.Trp478X	8173	3307 (40%)
29F	Blood	*STXBP1* (NM_003165)	chr9:130440783 G>A; c.1433G>A; p.Trp478X	2880	1 (0%)
29M	Blood	*STXBP1* (NM_003165)	chr9:130440783 G>A; c.1433G>A; p.Trp478X	10,021	2 (0%)
33	Blood	*KCNQ2* (NM_004518)	chr20:62076109 C>T; c.593G>A; p.Arg198Gln	15,892	5943 (37%)
33F	Blood	*KCNQ2* (NM_004518)	chr20:62076109 C>T; c.593G>A; p.Arg198Gln	17,410	7 (0%)
33M	Blood	*KCNQ2* (NM_004518)	chr20:62076109 C>T; c.593G>A; p.Arg198Gln	18,189	7 (0%)
35	Blood	*STXBP1* (NM_003165)	chr9:130438970-130438970 delC; c.1297delC; p.P433fs	4055	1599 (39%)
35F	Blood	*STXBP1* (NM_003165)	chr9:130438970-130438970 delC; c.1297delC; p.P433fs	1724	9 (0.5%)[c]
35M	Blood	*STXBP1* (NM_003165)	chr9:130438970-130438970 delC; c.1297delC; p.P433fs	5557	39 (0.7%)[c]
39	Blood	*CDKL5* (NM_003159)	chrX:18622719 C>T; c.1675C>T; p.Arg559X	1020	661 (65%)
39F	Blood	*CDKL5* (NM_003159)	chrX:18622719 C>T; c.1675C>T; p.Arg559X	2652	4 (0%)
39M	Blood	*CDKL5* (NM_003159)	chrX:18622719 C>T; c.1675C>T; p.Arg559X	1689	1 (0%)
44	Blood	*ARX* (NM_139058)	chrX:25025525 C>T; c.1151G>A; p.Arg384His	575	559 (97%)
44F	Blood	*ARX* (NM_139058)	chrX:25025525 C>T; c.1151G>A; p.Arg384His	841	1 (0%)
44M	Blood	*ARX* (NM_139058)	chrX:25025525 C>T; c.1151G>A; p.Arg384His	646	13 (2%)

Abbreviations: F, father; M, mother.

[a,b]Both total coverage and the alternate-allele reads were calculated according to the amplicon-based deep sequencing results.

[c]It was supposed to be sequencing error as mutant allele frequency was lower than 1% and it was identified in both of the parents.

et al., 2012) since the first case was reported by Dilena et al. (2015). Meanwhile, the invalid efficacy of LEV to epilepsy also has been reported in a patient carrying *STXBP1* mutation (Deprez et al., 2010; Li et al., 2016; Romaniello et al., 2013). In our cohort, LEV was prescribed to four patients with DNMs in *STXBP1*. The AEDs for the four patients before LEV treatment was shown in Table S6. It showed when the dosage of Levetiracetam was increased to 50 mg/kg/ day, three patients (Figure 2, Patients 31, 35, and 51) except for Patient 29 showed seizure-free within 40 days (range: 3–40 days). Consist, they all presented normal EEG within six months (range: 0.2–6 months). Further EEG interview still showed normal. Patient 29, who carries second DNM in *DIAPH3* beside *STXBP1*, presented intractable seizures and abnormal EEG even with the maximum dosage of LEV for three months. Our pilot retrospective analysis of LEV

therapy proved that LEV is effective for Chinese patients with *STXBP1* mutation.

4 | DISCUSSION

With the rapid development of sequencing technology, epilepsy panels or WES are now widely used as a first-tier genetic diagnostic strategy for pediatric EE. The diagnostic yield varies from 10% to 48.5% depending on the number of target genes (Carvill et al., 2013; Helbig et al., 2016; Lemke et al., 2012; Lindy et al., 2018; McTague et al., 2015; Trump et al., 2016). The diagnostic yield varies according to the age of onset also. For example, the yield is much higher among those in whom onset occurs under 2 months of age compared with those with onset <2 years (39% vs. 14%) (Trump et al.,
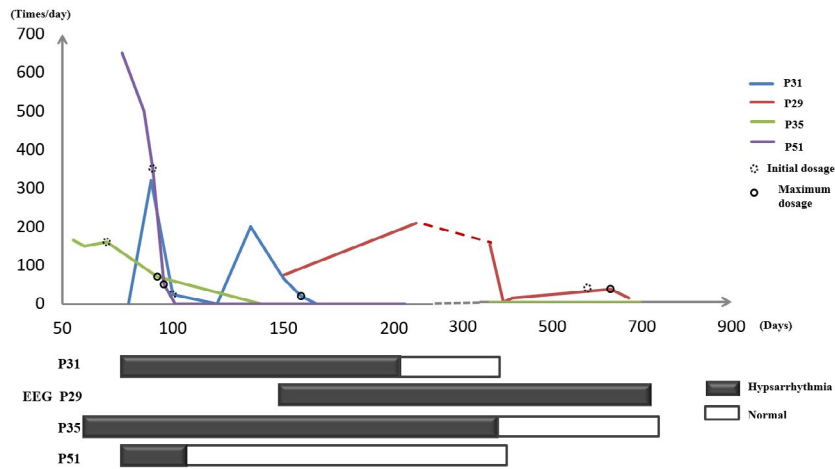
FIGURE 2 IS episode and treatment of Levetiracetam for four IS patients with *STXBP1* DNM. Different lines were used to represent the developmental trajectory of IS attacks in four patients with STXBP1 DNM. The *X*-axis represented the review point of EEG, treatment after birth, and the *Y*-axis represented the number of daily episodes of IS attack. Levetiracetam was gradually increased from 10 to 20 mg/kg/day (initial dosage, dashed circle) to 50 mg/kg/day (maximal dosage, solid circle). The progresses of their electroencephalograms (EEG) were illuminated different rectangles during 1.5 years after birth (black for hypsarhythmia and white for normal EEG)

2016). The diagnostic yield increased to 43% or 52% when the onset was in the neonatal period (Helbig et al., 2016; Trump et al., 2016).

The diagnostic yield of Chinese early-onset EE has previously been explored (Miao et al., 2018; Wang et al., 2019; Yang et al., 2018; Zhang et al., 2016). One recent publication on a Chinese pediatric epilepsy cohort showed that the genetic diagnostic rate was 26.7% (Yang et al., 2018). However, a similar study had not been carried out for an IS cohort. ISs occur in the first year after birth, so identifying the diagnostic yield and genetic spectrum of Chinese IS patients would improve diagnostic logistics. In this study, we revealed that the diagnostic yield of a Chinese undiagnosed IS cohort using 153-gene target sequencing was only 8.7%, which is much lower than those of Western IS/LGS and previous Chinese EE cohorts. We assumed that the true contribution of all monogenic disorders in this Chinese IS cohort could have been underestimated because all recruited patients had suffered diagnosis odyssey before being referred to Beijing Children's Hospital. More important, we strictly followed the ACMG guidelines to interpret the pathogenicity of each variant and excluded all uncertain variants from the monogenic diagnostic yield (DNM in *DIAPH3* and *CACNA1A*). In addition, for the inherited variant, although negative family history was recorded, the reduced penetrance of some inherited deleterious variants cannot excluded because we did not evaluate parents' phenotypes in detailed, and this also explained the low diagnostic yield in our IS cohort.

As noted above, recent studies using trio-based exome sequencing have confirmed that DNMs are a major etiological factor in Caucasian patients with EE (Allen et al., 2013; Carvill et al., 2013; Claes et al., 2001; Epi4K Consortium, 2016; EuroEPINOMICS-RES Consortium et al., 2014; Saitsu

et al., 2008; Shen et al., 2016; Trump et al., 2016; Yu et al., 2019). The 356 trio-based WES data of the Epi4K Consortium and Epilepsy Phenome/Genome Project showed that at least 12% of individuals with ISs or Lennox–Gastaut syndrome have a definitive disease-causing DNM (EuroEPINOMICS-RES Consortium et al., 2014). They also revealed that the top genes with recurrent DNMs are *SCN1A*, *STXBP1*, *GABRB3*, *CDKL5*, *SCN8A*, *SCN2A*, *ALG13*, *DNM1*, and *HDAC4* in descending order of frequency. Study of another Western early-onset seizure cohort proved that DNMs in *SCN2A* are predominant (10/400), followed by those in *CDKL5* and *STXBP1*, as revealed by sequencing of 46 genes (Trump et al., 2016). In contrast, a study of a medium-sized cohort of Western infants with EE (*n* = 500) with the sequencing of 65 genes showed that *CHD2* and *SYNGAP1*, rather than *SCN2A*, rank as hotspot genes with DNMs, accounting for 2.2% of referred patients (Carvill et al., 2013).

DNMs have been sporadically reported in Chinese EE cohorts (Miao et al., 2018; Xie et al., 2019; Zhang et al., 2016). However, neither the yield nor the architecture of DNMs has been distinctly characterized due to the small sample sizes. In our study, we revealed that pathogenic DNMs contributed to the causation of 8.3% (24/289) of Chinese patients with IS. This yield of DNM was similar to the yield (7.6%) of one Caucasian IS study with target sequencing (Muir et al., 2019), but lower than that of Epl4K Consortium (12%) with WES (EuroEPINOMICS-RES Consortium et al., 2014). More important, we found that the DNMs of dominant genes accounted for 100% of the monogenic diagnostic yield of ISs, indicating that DNMs of dominant genes rather than biallelic mutations of recessive genes contribute to ISs in this Chinese cohort.

The top-ranked genes with DNM in the Chinese IS cohort are *CDKL5* and *STXBP1*, rather than *SCN1A* found in

Caucasian EE cohorts (EuroEPINOMICS-RES Consortium et al., 2014; Trump et al., 2016), which accounted for 3.1% of cases in our IS cohort. Meanwhile, five of the DNM genes identified in the Caucasian EE cohort (*STXBP1*, *CDKL5*, *SCN2A*, *GABRB3*, *SCN8A*) were also detected in this Chinese IS cohort (EuroEPINOMICS-RES Consortium et al., 2014). The pathways/network of our DNMs are enriched in neurotransmission, similar to the network of DNMs in the Caucasian early-onset EE cohort (McTague et al., 2015), supporting the overlapping mechanism of ISs between different ethnic groups. Our study also supports the findings of Zhang et al., who identified *CDKL5* as the most common DNM-affected gene in their small Chinese early-onset EE cohort (17 genes, 175 patients), and DNMs in *CDKL5* accounting for 13.1% of tested patients (23/175) (Zhang et al., 2016). Moreover, in 2018, with a medium-sized Chinese early-onset epilepsy cohort, Yang identified some hotspot genes with recurrent variants (*ABCC8*, *CDKL5*, *DEPDC5*, *KCNQ2*, *MECP2*, *MUT*, *PCDH19*, *PRRT2*, *SCN1A*, *SCN2A*, *STXBP1*, and *TSC2*) (Yang et al., 2018). Notably, they found *KCNQ2* and *STXBP1* mutations were enriched in the neonatal group, but *SCN1A* mutation significantly occurred in the first-year group. Unfortunately, Yang did not perform inheritance analysis for these hotspot genes. In contrast to Yang's work, we completed parental validation for each pathogenic variant. We confirmed that their three genes (*CDKL5*, *STXBP1*, *SCN2A*) in neonatal group are hotspot genes with DNM in our IS patients. Inversely, none of *SCN1A* mutation was detected in our IS cohort, this suggested that *SCN1A* is not major causal gene carrying DNM in Chinese IS patients. Recently, another study of a large Western cohort also confirmed 100% DNM rates of epilepsy genes in *CDKL5*, *STXBP1*, *SCN8A*, *GABRA1*, and *FOXG1*, which is higher than the rates for *SCN2A* (90%), *GRIN2A* (88%), *KCNQ2* (82%), and *SCN1A* (76%) (Lindy et al., 2018). Considering the above, we assumed that the unidentified mutations in *CDKL5*, *STXBP1*, and *SCN2A* in Yang's Chinese cohort should be DNMs.

Recently, both germline mosaicism and somatic mosaicism have also been reported as important mechanisms for early-onset EE (Masliah-Plachon et al., 2010). Examples of somatic mosaicism include *KCNQ2* mutations in neonatal EE (Milh et al., 2015), *CDKL5* mutations in West syndrome (Jdila et al., 2017; Kato et al., 2015; Masliah-Plachon et al., 2010), paternal gonadal mosaicism of *SCN1A* mutations in Dravet syndrome (Depienne et al., 2010; Morimoto et al., 2006), and *STXBP1*-related Ohtahara syndrome (Saitsu et al., 2010). Hence, through deep sequencing, we identified that 22.7% (5/22) of the "*de novo* mutations in Sanger sequencing" are not really DNMs.

A previous study showed that carriers or patients with the mosaic mutation have milder symptoms than those with germline mutations, and that there is a positive relationship between mutant ratio and affected status (Stosser et al., 2017).

For example, a low somatic mutation level (16.9%) in *STXBP1* was associated with normal neurodevelopment (Saitsu et al., 2010), *SCN1A* mosaic mutation level was directly related to the severity of Dravet syndrome (Depienne et al., 2010), and patients with 20%–30% mutant ratio in *KCNQ2* presented with mild neonatal epilepsy but normal neurological development (Milh et al., 2015). As seen in our data, the patients with the somatic mutation, on either *CDKL5* or *STXBP1*, showed lower seizure attacks compared with ones with the germline mutation. However, due to limit sample size, further validation study with more Chinese IS patients carrying different somatic frequency in identical gene is needed.

A number of studies have shown that the seizure symptom of IS patients, carrying either null or missense *STXBP1* mutation, can be controlled by levetiracetam (Dilena et al., 2015). One Chinese study described the efficacy of levetiracetam on early-onset EE in 7 patients with DNM of *STXBP1* (Li et al., 2018). Among them, four patients showed good response to LEV alone or in combination with other routine AEDs, and were free from seizure for 4–18 months, and one patient showed reduced seizure (twice a year). Besides, in a recent study of 15 Chinese children with *STXBP1* mutations, a good response to levetiracetam in six of 8 children treated (Cao et al., 2020). Consistent, three of four patients with DNM of *STXBP1* in our study, also showed good response to LEV alone or in combination with other routine AEDs. Our study provides more evidence that levetiracetam can specifically reverse the *STXBP1*-related IS symptom regardless of null or missense mutation. However, as per the clinical phenotype of Patient 29 with a second DNM that we reported in this paper, modifying factors may nullify the efficiency of levetiracetam for some cases with *STXBP1* mutation. The clinical phenotype also influences the efficiency of Levetiracetam. In the future, more cases with *STXBP1* mutation and a second pathogenic mutation are needed to confirm the factors modifying treatment efficacy.

In conclusion, our study confirmed the contribution and genetic spectrum of DNMs, with an 8.3% yield in a Chinese IS cohort. Somatic mutations underlie the origin of 22.7% of DNMs in IS patients. Treatment with levetiracetam improved the prognosis of *STXBP1*-related ISs.

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ETHICAL COMPLIANCE

This study was approved by the Ethics Committee of Chinese People's Liberation Army General Hospital and informed consent for publication was obtained from all parents/legal guardians for case presentation and publication.

CONFLICT OF INTEREST

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

XLC and LZ designed the work. XLC finished final pathogenic interpretation for SNVs/CNVs and most of intellectual revision for manuscript. LL and FL are responsible for target gene design, data acquisition and manuscript drafting. FL, HX and YZ performed most of bench works including the target sequencing, amplicon-based deep sequencing, paternity testing, real-time PCR and aCGH experiment. LL and ZL performed most of day works including bioinformatics analysis, primer design, public mutation database researching and other statistical analyses. QW, QL, YW and MZ all were responsible for clinical works including patient recruitment, clinical data collection, patient review and treatment follow-up. PJ offered some genetic suggestion and language edition. XDC gave key suggestions for design and manuscript writing. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

All variant data supporting the conclusions of this manuscript were submitted to DECIPHER database.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.