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First report of Tunisian patients with *CDKL5*-related encephalopathy

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First report of Tunisian patients with CDKL5-related encephalopathy

Objective:

Mutations in the cyclin-dependent kinase-like 5 gene (CDKL5) are associated with a wide spectrum of clinical presentations. Early-onset epileptic encephalopathy (EOEE) is the most recognized phenotype. Here we describe phenotypic features in 8 Tunisian patients with CDKL5-related encephalopathy.

Methods:

We included all cases with clinical features consistent with CDKL5-related encephalopathy: infantile epileptic spasm, acquired microcephaly, movement disorders and visual impairment. We collected data about seizure types, electroencephalogram, magnetic resonance imaging and metabolic analysis. The diagnosis of CDKL5 mutation was made thanks to Sanger sequencing with an ABI PRISM 3100-Avant automated DNA sequencer using a Big Dye Terminator Cycle Sequencing Reaction Kit v1.1. and Next Generation Sequencing (NGS) since the development of a gene panel responsible for

DEE within the framework of “Strengthening the Sfax University Expertise for diagnosis and management of epileptic encephalopathies”.

Results:

We collected 4 boys and 4 girls aged meanly 6-years-old with confirmed mutation on CDKL5 gene. Overall, we identified 5 de novo CDKL5 mutations including three Frameshift mutations; one missense mutation and a splicing variant.

The mean age at first seizure onset was 4 months. The first seizure type was infantile epileptic spasm (4/8) followed by tonic (2/8) and myoclonic seizures (2/8). Out of 8 cases, 4 exhibited two stages epileptic course while epilepsy in 3 other patients progressed on three stages. Regarding development, most cases (6/8) had psychomotor retardation from the start whilst the two others showed psychomotor regression with the onset of seizures. Additional clinical features included visual impairment (7/8), tone abnormalities (7/8), stereotypies (7/8) and acquired microcephaly (6/8).

Significance:

Our present report delineates an unusual phenotype of CDKL5-related encephalopathy with male gender predominance and delayed onset epilepsy. It interestingly described new phenotypic features and uncommon benign developmental profiles in boys, different patterns of CDKL5-epilepsy, neuroimaging findings and CDKL5 mutational spectru

Introduction:

The cyclin-dependent kinase-like 5 (CDKL5) _also known as STK9_ belongs to a small family of five distinct Ser/Thr protein kinases, which contribute to signaling pathways basic for cell biology. The inactivation of the X-linked *CDKL5 gene* _or its mutations_ was responsible for a wide spectrum of clinical phenotypes grouped under the umbrella of CDKL5 deficiency disorder (CDD)[1]. The most recognized phenotype of *CDKL5*-related developmental and epileptic encephalopathy (DEE) is characterized by a 3-stage evolution of epilepsy with the onset of epilepsy before three months of age (stage 1), later progression into infantile epileptic spasm syndrome (IESS) (stage 2) and resulting ultimately in multifocal refractory epilepsy (stage 3) [2–4]. Epilepsy occurs in the setting of delayed psychomotor development and associates Rett-like features such as hand stereotypies and deceleration of head growth [2]. Despite the association of *CDKL5* mutations with these well-known syndromic clusters, recent publications continue to provide phenotypic data different from previous descriptions, leading to a broadened spectrum of *CDKL5*-related disorders [2–6]. Through this paper, we aim to depict the phenotypic specificities of Tunisian *CDKL5* cases.

Materials and methods

A. Patients and data collection:

Files of Patients with a follow-up at the Child neurology department of Hedi Chaker Sfax university hospital for Epileptic Encephalopathy (EE) beginning before the age of 1 year with neurodevelopmental impairment preceding or following the seizure onset, were retrospectively reviewed. Electro-clinical phenotyping was based on several EEGs coupled with video during the follow-up period, and the patient assignment was made according to the 2022 ILAE classification for seizures and epileptic syndromes [7–9]. All patients had brain imaging and metabolic screening before their enrollment for genetic analysis. Patients with onset EE before the age of 1 year, and clinical features suggestive of *CDKL5* mutation, including acquired microcephaly and involuntary movement, were proposed for targeted gene analysis using Sanger Sequencing. Since the development of a gene panel for DEE genetic assessment _including the *CDKL5* gene_ at Sfax University within the framework of "Strengthening the Sfax University Expertise for diagnosis and management of epileptic encephalopathies" (SEED project), CDKL5 gene sequencing was continued via Next Generation Sequencing (NGS) and SEED panel. Those whose neuroimaging showed major structural or signal abnormalities enough to explain the patient clinical picture or metabolic screening allowed the diagnosis of alternate inborn error of metabolism did not take part in genetic screening.

B. Molecular Methods

Blood samples were obtained from the children and their parents after informed consent. Genomic DNA was extracted from blood leukocytes by applying phenol-chloroform standard procedures. Polymerase Chain Reaction (PCR) was completed with amplification of the CDKL5 gene including 20 exons (2-21) and their exon-intron boundaries in all patients. PCR products were purified for mutation analysis. CDKL5 gene sequencing was performed by Sanger sequencing with an ABI PRISM 3100-Avant automated DNA sequencer using a Big Dye Terminator Cycle Sequencing Reaction Kit v1.1. ~~Since the development of a panel in Sfax University responsible for DEE including the CDKL5 gene within the framework of "Strengthening the Sfax University Expertise for diagnosis and management of epileptic encephalopathies" (SEED project), CDKL5 gene sequencing was continued via Next Generation Sequencing (NGS) and SEED panel~~. The SEED panel was designed using Design Studio to create a custom target enrichment library design (San Diego, CA 92122 USA). The design was based on GRCh37/hg19 reference sequences, with target sources obtained from the RefSeq

database. In this custom design, all coding exons were targeted involving 25 bp of the flanking intronic sequence of 116 genes. Genes were selected according to their relevance in DEE based on previously published genetic studies. The MiSeq Reporter software settings (Illumina) were adjusted to generate VCF files for index reads. VarAFT was used for variants annotation and filtering [10]. In addition, we examined the pathogenicity of the different variants following the ACMG (American College of Medical Genetics and Genomics) standards and guidelines [11]. Moreover, the Allele Frequency in the Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/) [12] was invested to assess the variant's frequency. Furthermore, we used a Human splicing finder to detect the impact of splicing variants on splicing signals [13]. This study was approved by the ethics committee of the Sfax Region.

Results

Demographic findings

Overall, the genetic analysis allowed the diagnosis of *CDKL5*-related DEE in 8 out of 44 analyzed patients (18.8%). Sanger sequencing, the technique used at the beginning of the study in 20 patients, identified the pathogenic variant of *CDKL5* mutation in six patients (30%) (respectively patients 1,2,3,5,6, and 7). Later, NGS using the developed panel was applied to locate *CDKL5* mutation in two other patients (Patients 4, 8) among the remaining 24 patients (8.3%) (**Table 1**). They were 4 boys and 4 girls (SR male/female = 1) with a mean age at the last visit of 9 years old (range 1.75 to 17.33 years). The mean age at the first visit to the child neurology department was 14.37 months (range 2 to 66 months) with a median delay to the first visit of about 20 days (ranging from 0 to 69 months). Our patients have a mean follow-up period of 7.8 years.

Three patients had consanguineous parents (P4, P5, and P6) and a family history of epilepsy (P1, P3, and P6). One patient (P6), whose sister also suffered from DEE, displayed severe epilepsy and developmental delay. Unfortunately, the sister died before getting a blood sample for genetic screening. ~~Such an approach if it was within reach would have helped to explain how genetic risk factors might modify the phenotype.~~

Molecular findings

Five pathogenic CDKL5 variants (NM_003159.2) were identified in the 8 patients in our cohort: the previously reported missense variant c.616 G>A in exon 9 (c.616 G>A; p.Asp206Asn), the frame-shift

reported variant in exon 19 (c.2788insG; p.Glu930GlyfsTer9) [14,15]. A novel non-sens variant in exon 12 (c.1910_1911ins19nt; p.Leu644Ter), a novel frame-shift variant (c.149delA; p.Asn50MetfsTer26) in exon 4, and a novel splicing variant (c.2153-1G>A) in exon 15 (**Table1**). All the variants detected were de novo in the patients since they were absent in their parents. The missense variant c.616 G>A in exon 9, was identified in a mosaic state in 3 boys (P1, P2, and P3) who were previously described [15], and in a heterozygous state in one girl (P5). The variant substitutes the highly conserved aspartate residue (Asp) with an asparagine residue (Asn) in position 206 (p.Asp206Asn) leading to conformational changes in different regions of the protein catalytic domain [15]. The c.2788insG variant in exon 19 identified in patient 6 resulted in a frame-shift mutation with a consequent premature stop codon in the C- terminal domain (p.Glu930GlyfsTer9). These changes alter protein functioning and disturb the dynamic regulation of isoform levels especially hCDKL5_5 and hCDKL5_1 during pre and postnatal neurodevelopment[14]. Variant c.1910_1911 ins 19nt in exon 12 (p.Leu644Ter) is also caused by the insertion of 19 nucleotides in exon 12 giving rise to a truncated protein in the C-terminal domain. The frame-shift variant c.149delA (p.Asn50MetfsTer26) leads to a premature stop codon and the production of a truncated non-functional protein devoid of its catalytic domain. The last one is a splicing variant affecting a canonical region (c.2153-1G> A) predicted to alter a wild-type acceptor site according to Human Splicing Finder prediction tool. The five variants described in this study are absent from control population databases (gnomAD). In addition, the four truncating variants (c.149delA ; c.2788insG ; c.1910_1911 ins 19nt; c.2153-1G> A) are classified as “Likely Pathogenic” according to the ACMG classification (**Table 1**).

Clinical Findings

At presentation, 6 cases (2 boys and 4 girls) (P3, P4, P5, P6, P7, and P8) had a global developmental delay from the start while the two remaining boys (P1 and P2) regressed with the onset of epilepsy. The main findings on examination at the first visit were poor eye contact (7/8) despite normal ophthalmologic exam, and severe tone abnormalities (7/8). Acquired microcephaly and dysmorphism were found in 6/8 and 4/8 of our patients, respectively. Common observed dysmorphic features in our patients included: a narrow forehead, low hairline, hypertelorism, deep-set eyes, ogival palate, and micrognathia. Two patients (P5 and P6) had mild hearing loss due to retro-cochlear auditory pathways impairment, for which no genetic screening was necessary. During follow-up, 4 patients (3 boys and 1 girl) improved their gross motor milestones with independent sitting position at a mean age of 30.5 months (P2 and P4) and independent walking at a mean age of 7.5 years (P1 and P3). All

of them (P1, P2, P3, and P4) remained deeply impaired in cognitive skills. Other symptoms like abnormal involuntary movements, including chorea in 3 patients (P2, P3, and P7) and non-epileptic myoclonus in one patient (P7), as well as hand stereotypies (P1, P2, P3, P4, P5, and P7), were noted on follow-up examination with a mean delay of 15 months and 33 months, respectively.

Epilepsy and EEG findings:

The first epileptic seizure occurred between 3 weeks and 8 months of age with a mean age at onset of 4 months. Age at onset of epilepsy was slightly more delayed in boys (4.9 months) than in girls (3.32 months). In the beginning, seizure types included epileptic spasms (ES) in 3 boys and 1 girl (P1, P2, P3, and P8), tonic seizures (TS) in 2 girls (P5 and P7), and myoclonic seizures in 2 cases, 1 boy and 1 girl (P4 and P6). Myoclonic seizures seemed to have earlier onset mean age (1 month) compared to TS (2 months) and ES (6.75 months). Awake and sleep EEG recordings in patients with ES at onset found interictal hypsarrhythmia in all 4 cases (P1, P2, P3, and P8) defining Infantile Epileptic Spasm Syndrome (IESS). During evolution, our patients showed two different epilepsy courses with the first made of a two-stage course in 4 patients (P1, P2, P3, and P4), and the second made of a three-stage course in 3 patients (P5, P6, and P7). The outcome of patient 8 (P8) _who had a short follow-up period limited to 12 months_ needs further electro-clinical monitoring.

The critical time point determining the shift from one stage to another corresponded to the moment of marked changes in electro-clinical characteristics with the appearance of a new seizure type or significant increase in EEG interictal activity under optimal and well-conducted antiepileptic treatment. The different stages of seizures storm inconstantly alternated with periods with good seizures-control, also called seizure-free periods.

Three children (P1, P2, and P3) among those who initially presented with IESS and one child (P4) out of the two patients with a myoclonic seizure at onset exhibited a two-stage epilepsy course. They displayed a different seizure type after a mean seizure-free period of 16.75 months (ranging from 4 to 32 months) and a mean time from epilepsy onset of 32 months (ranging from 12 to 68 months). Both, patients 1 (P1) and 3 (P3) progressed to tonic seizures that responded well to ASMs, while patient 2 (P2) developed refractory multifocal epilepsy. Patient 4 (P4) evolved to IESS without hypsarrhythmia.

The two patients 5 (P5) and 7 (P7) who initially presented with TS, and one child (P6) with a myoclonic seizure at onset displayed a three-stage epilepsy course. Patient 5 (P5) had absence seizures as a

second seizure type for a long time before he additionally presented focal motor tonic and clonic seizures defining a Lennox Gastaut Syndrome (LGS), which was responsive to ASMs. Patient 7 (P7) developed IESS with hypsarrhythmia, then refractory multifocal epilepsy. Patient 6 (P6) experienced IESS as a second epilepsy course, and subsequently, she evolved to her third stage in the form of intractable LGS.

In patients with a three-stage epilepsy course, the second seizure type occurred after a mean seizure-free period of 5.3 months (ranging from 3 to 9 months). The mean delay between the second (intermediate) and the third (ultimate) epilepsy course stage was 40 months (ranging from 10 to 96 months). This group included more girls (P5 and P7) than boys (P6), all of whom showed a poor developmental outcome. Further details regarding electro-clinical data are available in **Table 1**.

Brain MRI findings:

Brain MRI was normal in 4 cases (P1, P3, P4, and P5) and demonstrated minor structural changes in the remaining patients (P2, P6, P7, and P8) with frontotemporal cortex atrophy in 4 cases (P2, P6, P7, and P8) associated to thin corpus callosum in 2 cases (P2 and P7), and delayed myelination in patient 8 (P8) (**Figure 1**). Neuroimaging data are summarized in **Table 1**.

Discussion:

The present study categorized CDKL5 as a prominent DEE-related gene whose mutation was identified in nearly a fifth of our whole population. It identified five de novo CDKL5 gene mutations. Among these, three new variants constituted a unique finding of the present study. In addition, this paper drew epilepsy courses and key clinical features associated with CDKL5–related DEE. Indeed, CDKL5 mutation was unexpectedly found in 4 boys, and showed delayed onset epilepsy [14,15]. Until 2018, only 25 mutations in *CDKL5* were reported in boys, as compared with 131 reported in females [16]. These data gave evidence for female predominance. CDD is linked to the X chromosome and affects the female rather than the male gender (SR: 4/1) whatever the phenotype. Indeed, males with germline variants are devoid of normal *CDKL5* gene and run a lethal risk during fetal life [5,17]; while survivors exhibit more severe disease courses. In contrast, phenotype severity in females was related to X chromosome inactivation at birth [16,17,18]. In our cohort, we explain the relatively milder epilepsy phenotype in three boys by the presence of the mutation at a mosaic state in 3 cases [15]. At the time of their first evaluation, our cases' mean age was 14.37 months (ranging from 2 to 66 months); this greatly exceeds what has been reported in a previous case series study about 8 boys

with *CDKL5*-related DEE whose ages at first examination ranged from 2 to 168 months [4]. According to another report, the median age at the first visit was more delayed rising up to 4.7 years in females and 5.2 years in boys but the author included patients with DEE as well as those with Rett phenotype [19].

Eighty percent of patients with CDD present with developmental delay, this is almost what was depicted in our series where 6 out of 8 cases (75%) had a global developmental delay from the start. On the other hand, regression is reported in rare cases with frequent seizures or epileptic encephalopathy as it happened in two of our reports [2–4,18–25]. In terms of clinical findings, our data met those commonly described in the literature. Indeed, cortical visual impairment evidenced in most children as poor eye contact regardless of normal ophthalmologic exam stands for at least 75% of individuals among numerous series [3,22–29]. Accordingly, severe tone abnormalities present in most of our patients are a well-recognized feature of CDD [5,23,30]. Auditory pathways impairment seen in 2 of our 8 reports have not yet been reported to the best of our knowledge. Nonetheless, further genetic analysis assessing deafness causing gene mutations was not required. In fact, our patients displayed isolated retro-cochlear damage on Brain Evoked Response, which was an uncommon finding for hearing impairment of a genetic origin [31]. Rett-like features including hand stereotypies occurred in 7 out of 8 patients after a median follow-up period of 33 months. This is consistent with the observed rate of around 80% in the literature where hand stereotypies appear since the first year of life and become more obvious over time [2,5,17,32,33]. Other movement disorders such as chorea noted in 3 cases in association with myoclonus in 1 case among our patient cohort were previously reported [34–36].

According to the literature, limitation in milestones attainment across different developmental areas concerned mainly communications and fine motor skills compared to achievements in gross motor skills. Moreover, when attained, they were significantly delayed [2,17,28]. In a recent publication assessing girls, independent walking was attained by 22–23% by 4 years and a half whilst only 16% of subjects could speak single words by 7 years of age [28]. In our study, among patients with sufficient follow-up period, just 1 girl and 1 boy acquired sitting positions at 13 months and 48 months respectively. Two boys (25%) were able to walk independently at respectively 7 and 8 years old while others (1 boy and 2 girls) remained bedridden. Only 2 cases (1 girl and 1 boy) were able to talk single words at 2 and 7 years respectively. Although males are known to have a severe neurodevelopmental profile, males achieved more developmental milestones than wait in the present study. A previous

study also reported 4 boys who acquired independent sitting positions and walking with limited communication skills. The author thus suggested a variability relative to developmental milestones attainment in males with the CDKL5. He then explained disagreement with the literature data by the limited size of male samples reported and the selection of severely affected boys for CDKL5 mutation screening [28].

Dysmorphic features described in 4 cases in our cohort (50 %) were observed in a small number of patients (5.7%) of previous reports [5,17,18]. Common observed facial features in our patients included: a narrow forehead, low hairline, hypertelorism, deep-set eyes, ogival palate, and micrognathia. Our findings were different from those describing subtle dysmorphic features including mostly broad or high forehead, deep-set eyes, deep philtrum, prominent lips, puffy phalanges, and tapered fingers [5,18,19,22,23,25,37]. Deceleration of head growth seen in 6 of our 8 cases (75%) exceeded the rate of 10% reported in the literature [3,5,19,21–25,34]. We explained this finding by the fact that acquired microcephaly was one of the selection criteria for Sanger Sequencing our study cohort.

Consistent with the findings of previous studies, ES was the leading seizure type (4/8) at onset epilepsy in our cohort and appeared in later disease stages in 3 other cases. Other seizure types included tonic (2/8) and myoclonic (2/8) seizures, too. The estimated frequency of ES at the onset of the disease is about 23% and occurred in about 81% at some point of the disease course [17,35,36]. According to the *CDKL5* Centers of Excellence (COEs), median epileptic spasm onset was 4 months of age ranging from 2 weeks to 36 months but the author did not specify if he considered all cases with spasms whatever the time of their occurrence [5]. Overall, the median age of epilepsy onset is 6 weeks with 90% onset by 3 months [5,19,32]. By contrast, our reports showed more delayed ES onset between 5 and 8 months while early onset seizures from 3 weeks to 3 months were more often tonic and myoclonic seizures with a mean age at onset epilepsy of 4 months (ranging from 3 weeks to 8 months) considering all seizure types. EEG at onset ES as the first seizure type demonstrated hypsarrhythmia in all cases. Among those who developed ES as a second seizure type, only 2 out of 3 displayed hypsarrhythmia on EEG. In fact, ES associated with CDD may occur without hypsarrhythmia with an EEG showing only a rare interictal epileptiform activity, or even normal [5,27]. During the follow-up period, our patients showed two models of epileptic seizure evolution thus leading to dividing them into two groups according to their epilepsy course. The second group (P5, P6, P7) displayed the characteristic three-stage electro-clinical epilepsy with onset at the latest 3 months of age. This typical progression was first described by Bahi-Buisson et al who paid attention to three stages with

the onset of epilepsy before three months of age (stage 1), followed by epileptic spasms (stage 2), and later multifocal refractory epilepsy (stage 3) [2,5,6,17,26,38–40]. Next, a subsequent two-stage epilepsy pattern was reported with a second stage made of hyper motor-tonic-spasms sequence occurring between 3.5 and 13 months (median 7.5 months), after a period of 1.5 to 11 months from onset epilepsy (stage 1) but the author did not precise first seizure type [2,5,6,17,26,38–40]. In the present study, the two-stage epilepsy course was also noted in P1, P2, P3, and P4. These latter presented with delayed ES between 5 and 8 months at onset in 3 boys (P1, P2, and P3) and myoclonic seizure at 40 days in 1 girl (P4) (stage 1) and ultimately developed polymorphous seizures (stage 2) including tonic (P1 and P3), multifocal seizures (P2) and tonic spasm (P4). These heterogeneous data arising from different clinical observations indicate the need for further studies with a greater sample size to improve our understanding of CDKL5-related epilepsy.

According to the COE cohort, seizure-free periods varied between 1 and 12 months for 32 % of families while only 13% of families estimated this period to be more than 12 months. They specified this honeymoon period typically occurs before 2 years of age, yet some can experience seizure-free periods later in childhood or into their teenage years [5]. These data are consistent with our results where patients had a median free-seizure period of around 11 months.

Regarding neuroimaging data, some researchers found no abnormalities on brain MRI [26] while others noticed mild frontal lobe atrophy in almost all patients [23]. This ascertainment was also seen in 4 of our 8 patients in association with thin corpus callosum in 2 cases, hypomyelination, and basal ganglia signal abnormalities carrying out additional characteristics for patients with CDD. Our study added three novel pathogenic variants to the previously set of published mutations and thus gave a continuation for earlier reviews. In that issue, most reported *CDKL5* mutations were also sporadic and considered as de novo with a very small number of recurrent variants [41]. More data about *CDKL5* mutation-related disorder according to literature are available in **Table 2**.

Although the phenotypes of our patients overlap at some points with those reported in the literature, we noted a few such as milder phenotypes in boys, hearing loss, and dysmorphic traits. Furthermore, our cohort showed phenotypic heterogeneity with inconsistent disease outcome and severity between patients even in those carrying the same mutation indicating probably epigenetic factors implication in the determinism of gene expression. This hypothesis may explain our failure and that of previous studies to establish any genotype-phenotype correlation [5,27,29]. Although some authors thought that frame-shift mutations in the C-terminal region cause more severe phenotypes as was

the case in our sixth (P6) and seventh (P7) reports; the relationship between genotype and phenotype is still ambiguous [36,42,43].

Conclusion:

Our data as well as those of the literature well demonstrated CDD is responsible for DEE. The present study interestingly described new phenotypic features such as hearing loss and peculiar dysmorphic traits. We also depicted more reports relative to unusual benign developmental profiles in boys carrying CDKL5 mutation, different patterns of CDKL5-epilepsy, neuroimaging findings, and CDKL5 mutational spectrum. However, we lacked information about CDKL5-related comorbidities such as sleep disorders, and respiratory and feeding problems. Consequently, a screen for CDKL5 mutations is mandatory in the setting of early onset epilepsy mainly IS and associated Rett-like features independently of associated unusual phenotypic findings. However, the fact that previous studies described CDKL5 in boys [44], and half of our sample individuals with CDKL5 mutations were male warrants the necessity of considering the analysis of CDKL5 in boys with DEE, too.

key bullet points:

- *CDKL5* gene should be considered as fundamental for a gene panel designed to assess DDE as *CDKL5* mutation was frequently associated to such phenotype.
- *CDKL5*-related DEE affected both girls and boys justifying considering CDKL5 mutation in boys with DEE, too.
- Understading epilepsy course associated with *CDKL5*-related DEE may help the development of a personalized management.

References:

1. Patrick A Eyers. A new consensus for evaluating CDKL5/STK9-dependent signalling mechanisms. *The EMBO Journal* **37***: e100848 (2018).* https://doi.org/10.15252/embj.2018100848.
2. Bahi-Buisson, N. & Bienvenu, T. CDKL5-Related Disorders: From Clinical Description to Molecular Genetics. *Mol. Syndromol.* **2**, 137–152 (2012).
3. Bahi-Buisson, N. *et al.* Key clinical features to identify girls with CDKL5 mutations. *Brain J. Neurol.* **131**, 2647–2661 (2008).

4. Mirzaa, G. M. CDKL5 and ARX mutations in males with early-onset epilepsy. 20 (2014).
5. Olson, H. E. *et al.* Cyclin-Dependent Kinase-Like 5 Deficiency Disorder: Clinical Review. *Pediatr. Neurol.* **97**, 18–25 (2019).
6. Sartori, S. *et al.* A novelCDKL5mutation in a 47,XXY boy with the early-onset seizure variant of Rett syndrome. *Am. J. Med. Genet. A.* **149A**, 232 (2009).
7. Rima Nabbout. *et al.* ILAE classification and definition of epilepsy syndromes with onset in neonates and infants: Position statement by the ILAE Task Force on Nosology and Definitions *Epilepsia* **63**,1349–1397 (2022).
8. Fisher, R. S. *et al.* Operational classification of seizure types by the International League Against Epilepsy: Position Paper of the ILAE Commission for Classification and Terminology. *Epilepsia* **58**, 522–530 (2017).
9. Scheffer, I. E. *et al.* ILAE classification of the epilepsies: Position paper of the ILAE Commission for Classification and Terminology. *Epilepsia* **58**, 512–521 (2017).
10. Desvignes, J.-P. *et al.* VarAFT: a variant annotation and filtration system for human next generation sequencing data. *Nucleic Acids Res.* **46**, W545–W553 (2018).
11. Richards, S. *et al.* Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet. Med. Off. J. Am. Coll. Med. Genet.* **17**, 405–424 (2015).
12. gnomAD. https://gnomad.broadinstitute.org/.
13. Desmet, F.-O. *et al.* Human Splicing Finder: an online bioinformatics tool to predict splicing signals. *Nucleic Acids Res.* **37**, e67 (2009).

14. Jdila, M. B. *et al.* A novel C-terminal truncated mutation in hCDKL5 protein causing a severe West syndrome: Comparison with previous truncated mutations and genotype/phenotype correlation. *Int. J. Dev. Neurosci. Off. J. Int. Soc. Dev. Neurosci.* **72**, 22–30 (2019).
15. Jdila, M. B. *et al.* Novel mutations in the CDKL5 gene in complex genotypes associated with West syndrome with variable phenotype: First description of somatic mosaic state. *Clin. Chim. Acta* **473**, 51–59 (2017).
16. Liang, J.-S. Phenotypic manifestations between male and female children with CDKL5 mutations. 7 (2019).
17. Jakimiec, M., Paprocka, J. & Śmigiel, R. CDKL5 Deficiency Disorder-A Complex Epileptic Encephalopathy. *Brain Sci.* **10**, 107 (2020).
18. Archer, H. L. *et al.* CDKL5 mutations cause infantile spasms, early onset seizures, and severe mental retardation in female patients. *J. Med. Genet.* **43**, 729–734 (2006).
19. Fehr, S. *et al.* The CDKL5 disorder is an independent clinical entity associated with early-onset encephalopathy. *Eur. J. Hum. Genet. EJHG* **21**, 266–273 (2013).
20. Fehr, S. *et al.* Functional abilities in children and adults with the CDKL5 disorder. *Am. J. Med. Genet. A.* **170**, 2860–2869 (2016).
21. Liang, J.-S. *et al.* CDKL5 alterations lead to early epileptic encephalopathy in both genders. *Epilepsia* **52**, 1835–1842 (2011).
22. Mei, D. *et al.* Xp22.3 genomic deletions involving the CDKL5 gene in girls with early onset epileptic encephalopathy. *Epilepsia* **51**, 647–654 (2010).
23. Olson, H. E. & Poduri, A. CDKL5 mutations in early onset epilepsy: Case report and review of the literature. *J. Pediatr. Epilepsy* **1**, 151–159 (2012).
24. Russo, S. *et al.* Novel mutations in the CDKL5 gene, predicted effects and associated phenotypes. *Neurogenetics* **10**, 241–250 (2009).

25. Sartori, S. *et al.* Pathogenic role of the X-linked cyclin-dependent kinase-like 5 and aristaless-related homeobox genes in epileptic encephalopathy of unknown etiology with onset in the first year of life. *J. Child Neurol.* **26**, 683–691 (2011).
26. Artuso, R. *et al.* Early-onset seizure variant of Rett syndrome: definition of the clinical diagnostic criteria. *Brain Dev.* **32**, 17–24 (2010).
27. Mefford, H. C. Phenotype to Genotype and Back Again. *Epilepsy Curr.* **20**, 88–89 (2020).
28. Fehr, S. *et al.* There is variability in the attainment of developmental milestones in the CDKL5 disorder. *J. Neurodev. Disord.* **7**, 2 (2015).
29. Demarest, S. T. *et al.* CDKL5 Deficiency Disorder: Relationship between genotype, epilepsy, cortical visual impairment and development. 17 (2020).
30. Fehr, S. *et al.* Seizure variables and their relationship to genotype and functional abilities in the CDKL5 disorder. *Neurology* **87**, 2206–2213 (2016).
31. Nicolas Michalski *et* Christine Petit. Central auditory deficits associated with genetic forms of peripheral deafness. *Hum Genet.*; **141**(3-4): 335–345 (2022). https://doi.org/10.1007%2Fs00439-021-02339-3**.**
32. Frullanti, E. *et al.* Analysis of the Phenotypes in the Rett Networked Database. *Int. J. Genomics* **2019**, 6956934 (2019).
33. Saitsu, H. *et al.* A girl with early-onset epileptic encephalopathy associated with microdeletion involving CDKL5. *Brain Dev.* **34**, 364–367 (2012).
34. Nemos, C. *et al.* Mutational spectrum of CDKL5 in early-onset encephalopathies: a study of a large collection of French patients and review of the literature. *Clin. Genet.* **76**, 357–371 (2009).
35. Wong, V. C.-N. & Kwong, A. K.-Y. CDKL5 variant in a boy with infantile epileptic encephalopathy: case report. *Brain Dev.* **37**, 446–448 (2015).

36. Zhao, Y. *et al.* Clinical features and gene mutational spectrum of CDKL5-related diseases in a cohort of Chinese patients. *BMC Med. Genet.* **15**, 24 (2014).
37. CDKL5 mutations in boys with severe encephalopathy and early-onset intractable epilepsy | Neurology. https://n.neurology.org/content/71/13/997.long.
38. Grosso, S. *et al.* Seizures and electroencephalographic findings in CDKL5 mutations: case report and review. *Brain Dev.* **29**, 239–242 (2007).
39. Klein, K. M., Yendle, S. C. & Harvey, A. S. A distinctive seizure type in patients with CDKL5 mutations: Hypermotor-tonic-spasms sequence. 5 (2011).
40. Melani, F. *et al.* CDKL5 gene-related epileptic encephalopathy: electroclinical findings in the first year of life. *Dev. Med. Child Neurol.* **53**, 354–360 (2011).
41. CDKL5 alterations lead to early epileptic encephalopathy in both genders. 8 (2011).
42. Bahi-Buisson, N. *et al.* Recurrent mutations in the CDKL5 gene: Genotype–phenotype relationships. *Am. J. Med. Genet. A.* **158A**, 1612–1619 (2012).
43. Kilstrup-Nielsen, C. *et al.* What We Know and Would Like to Know about CDKL5 and Its Involvement in Epileptic Encephalopathy. *Neural Plast.* **2012**, 728267 (2012).
44. Siri, B. CDKL5 deficiency disorder in males: Five new variants and review of the literature. 34.

Figure Legend

The figure shows delayed myelination on Brain MRI of patients 8 performed at the age of 8 months. Axial T2 sequences (A and B) demonstrated a slight hyper-signal through the median thalamus and rostral lenticular nuclei as well as subcortical and deep white matter, While these stated brain area did not show any signal changes on axial Tl sequences (C and D).

		Patient 1 (P1)*	Patient 2 (P2)*	Patient 3 (P3)*	Patient 4 (P4)	Patient 5 (P5)	Patient 6 (P6) **	Patient 7 (P7)	Patient 8 (P8)
Gender		M	M	M	F	F	M	F	F
Age at First visit (months)		16	8	5	2	66	5	4	9
Age at last visit (months)		208	76	149	28	186	132	72	21
Follow-up period (months)		192	68	145	26	120	127	68	12
Consanguinity		-	-	-	+	+	+	-	-
Family history of epilepsy		+	-	+	-	-	+ (DEE)	-	-
Clinical findings	Acquired microcephaly	-	+	-	+	+	+	+	+
	Abnormal movements	Hand stereotypies	Hand stereotypies Chorea	Hand stereotypies Chorea	Hand stereotypies	Hand stereotypies	-	Hand stereotypies Chorea Non epileptic myoclonus	Hand stereotypies
	Development course								
	Initial cognitive and motor development	normal, with regression at onset of seizures	normal, with regression at onset of seizures	Delayed	Delayed	Delayed	Delayed	Delayed	Delayed
	Language	2 words (7 years)	Babbling (4 years)	-	Single words (2 years)	-	-	-	-
	Independent Walking	7 years	-	8 years	-	-	-	-	-
	Epilepsy								
	Onset of epilepsy	6 months	8 months	5 months	40 days	3 months	3 weeks	1 month	8 months
	First type of seizure / epilepsy syndrome	ES / IESS	ES / IESS	ES / IESS	Myoclonic seizures	TS	Myoclonic seizures	TS	ES/IESS
	Seizure-free period	12 months	32 months	7 months	16 months	9 months	4 months	3 months	-
	Age at onset second seizure type	18 month	76 months	36 months	18 months	12 months	5 months	4 months	NA
	Type of seizures / epilepsy syndrome during evolution	FMTS	Multifocal seizures (FMTS and FMCS)	TS	Tonic spasm / IESS	Absence seizures	ES / IESS	ES / IESS	ES/IESS
	Age at onset third seizure type	-	-	-	-	108 months	15 months	18 months	-
	Type of seizures / epilepsy syndrome during evolution	-	-	-	-	FMTS, FMCS / LGS	Absence, TS / LGS	Multifocal seizures	-
	Epilepsy course	2 stages	2 stages	2 stages	2 stages	3 stages	3 stages	3 stages	-
	Response to ASMs (Seizure free)	+	-	+	No data	+	-	-	-
EEG findings	At onset During follow up	(1) Hypsarrhythmia (2) Normal (16 months)	(1) Hypsarrhythmia (2) Diffuse epileptiform abnormalities (58 months)	(1) Hypsarrhythmia (2) Focal epileptiform abnormalities (48 months)	(1) Ictal EEG: Diffuse polyspikes synchronous to myoclonus / Inter-ictal: normal (2) Inter-ictal : multifocal epileptiform abnormalities (18 months)	(1) Not available (2) Ictal EEG : FMCS synchronous to fronto-polar polyspikes discharges (186 months)	(1) Focal paroxysm on well-organized background activity (2) Hypsarrhythmia (7 months) (3) Diffuse irregular spike and wave (96 months)	(1) Not available (2) Hypsarrhythmia (14 months) (3) multifocal epileptiform abnormalities (29 months)	(1) Hypsarrhythmia (2) Inter-ictal : multifocal epileptiform abnormalities (12 months)
Brain MRI		**9 years:** Normal	**14 months:** fronto-temporal cortex atrophy + thin corpus callosum	**5 years:** Normal	**2 years 3 months:** Normal	**9 years 3 months:** Normal	**7 months / 11 years:** fronto-temporal cortex atrophy	**13 months*****: fronto-temporal cortex atrophy + thin corpus callosum	**8 months:** fronto-temporal cortex atrophy + Delayed myelination
CDKL5 Mutation		De novo c.616 G>A (E9) (p.Asp206Asn) at a somatic mosaic state			De novo c.149delA (p. Asn50MetfsTer26) at a heterozygote state	De novo c.616 G>A (E9) (p.Asp206Asn) at a heterozygote state	De novo c.2788insG (E19) p.Glu930GlyfsTer9	De novo c.1910_1911 ins 19nt (E12) (p.Leu644Ter) at a heterozygote state	c.2153-1G>A (E15) at a heterozygote state
ACMG classification of the mutation		VUS			LP	VUS	LP	LP	LP
Frequencies (gnomAD)		VNF			VNF	VNF	VNF	VNF	VNF

M: male, F: female, S: stage, E: exon, (+): yes, (-): no, ES: Epileptic Spasm, TS: Tonic Seizures, FMTS: Focal Motor Tonic Seizures, FMCS: Focal Motor Clonic Seizures, ASMs: anti-seizures medications, IESS: Infantile Epileptic Spasm Syndrome, LGS: Lennox Gastaut Syndrom, VNF: Variant Not Found, LP: Likely Pathogenic, VUS: Variant of Uncertain Significance

(*): indicates the patient has been previously reported in a separate publication (15).

(**): indicates the patient has been previously reported in a separate publication (14).

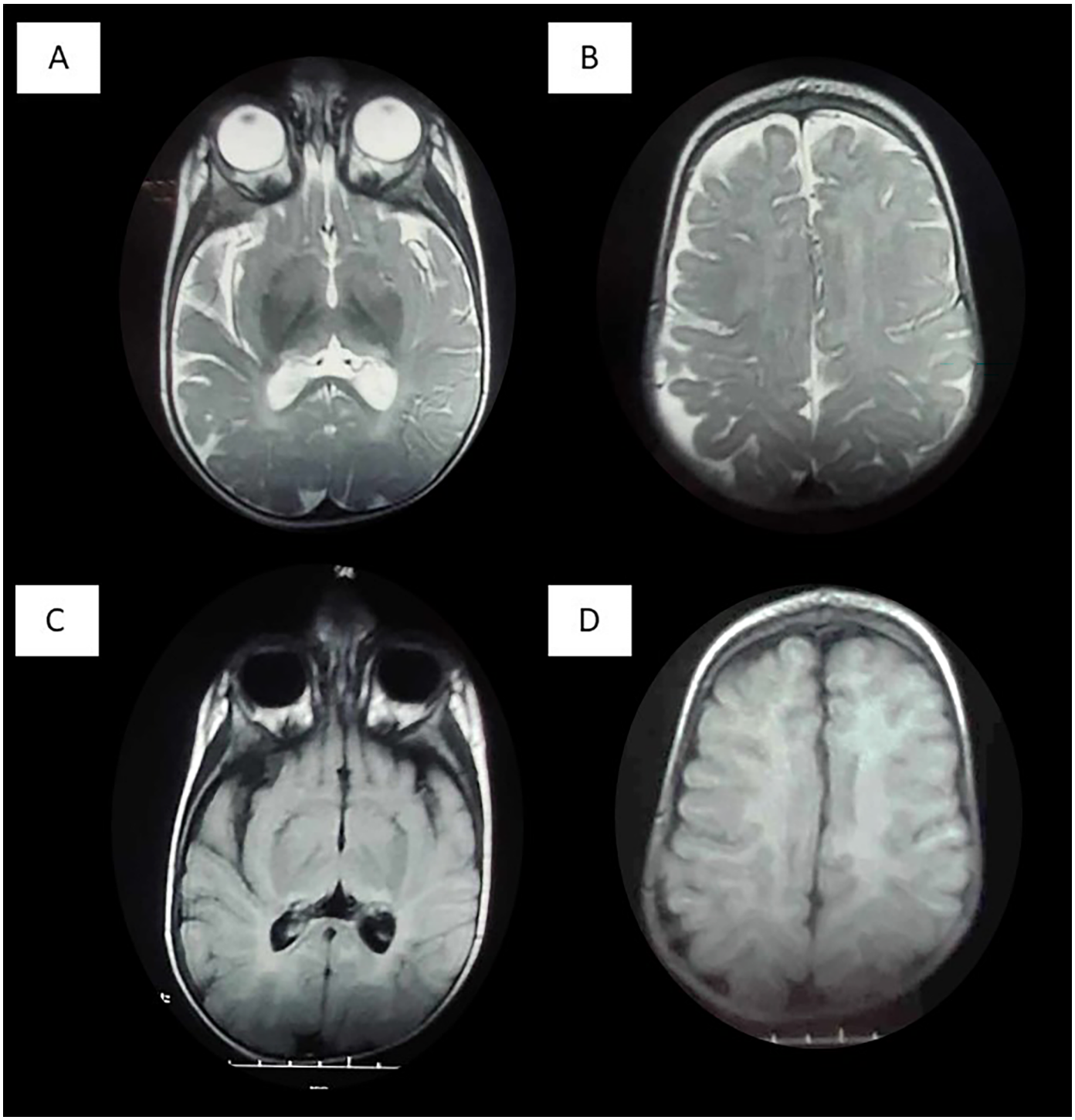
(***): Data according to CT scan

Table 1: Clinical, neuroimaging and genetic characteristics of patients with CDKL5 mutation

Study	Present study	Jao-Shwann Liang and al. 2011 [41]	Ying Zhao and al. 2014 [36]	Barbara Siri and al. 2021 [44]
Number	8	12	10	50
Age (Average / months)				
First visit	14.37 (2-66)	26 (6-55)	No data	No data
Last visit	108 (21-208)	No data	31.5 (5-60)	71 (5-252)
Gender (SR: Female/Male)	4 F / 4 M: 1	9 F / 3 M: 3	9 F/ 1 M: 9	5 new boys and review of 45 previously reported males
Physical Examination				
Acquired Microcephaly	75%	16.6%	100%	20%
Dysmorphism	50%	No data	No data	20%
Neurologic features				
Poor eye contact	87.5%	No data	No data	64%
Tone abnormalities	87.5%			
Axial hypotonia	87.5%	83%	100%	68%
Limb spasticity	75%	No data	No data	26%
Autistic features				
Stereotyped movements	87.5%	41.6%	90%	8%
Movement disorders	37.5%	No data	No data	46%
Development				
Speech	12.5% (single word)	0%	10% (single word)	No data
Limited hand skills	25%	No data	100%	2%
Sitting	37.5%	16.6%	70% (12.4months)	6%
Walking	25%	0%	10% (few steps)	4%
Epilepsy				
Age at onset (Average / months)	4 (3 w - 8 months)	1.5 (4 d - 6 months)	1.5 (10 d - 3.3 months)	5 (4 d – 132 months)
First seizure type	ES (50%); TS (25%); Myoclonic (25%)	ES (75%); Focal (25%); GTC (25%); Myoclonic (8.3%)	Focal (100%); ES (10%)	ES (38%); Focal and GT
Second seizure type	ES (62.5%); Focal motor seizure tonic (37.5%) clonic (25%); TS (25%) Absence (25%)	No data	ES (80%); GT (20%); Myoclonic (20%)	Myoclonic (36%)
Disease course	Two (50%) / Three-stage course (37.5%)	No data	Two-stage course	Three-stage course
Refractory EE to ASM	50%	91.6%	100%	96%
Seizure-free period	11 (3 - 32 months)	No data	No data	8% (2 w – 24 months)
EEG				
Hypsarrhythmia at onset	50%	No data	50%	16%
Hypsarrhythmia during follow-up	25%	No data	No data	22%
Brain MRI				
Fronto-temporal cortex atrophy	50%	100%	0%	28%
Thin corpus callosum	25%	0%	0%	10%
Delayed myelination	12.5%	0%	0%	0%
Vermian hypoplasia	0%	0%	0%	2%
Normal	50%	0%	100%	NA

Genetic				
Novel / recurrent mutation	4/0	6/2	9/1	46/4
De novo	4	58.3%	100%	96%
Mutation type	Frame-shift (3) Missense (1)	Nonsense (3) Frame-shift (3) Missense (2)	Micro-deletion (3) Insertion; Splicing (2) Nonsense; Missense (1)	Missense (2/5) Frame-shift; in-frame deletion; splice site (1/5)
Genotype-phenotype correlation	Seizure control in three cases with MS mutation	Seizure control in one patient with MS mutation	seizure control and in one patient with splicing mutation	Late onset epilepsy in one patient with MS mutation

Table 2: The table shows clinical phenotype, epilepsy course, neuroimaging features in patient with CDKL5 mutation as reported in the present and previous study



Zouari Fig_1.tif