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Key clinical features to identify girls with *CDKL5* mutations

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Mutations in the human X-linked cyclin-dependent kinase-like 5 (*CDKL5*) gene have been shown to cause infantile spasms as well as Rett syndrome (RTT)-like phenotype. To date, less than 25 different mutations have been reported. So far, there are still little data on the key clinical diagnosis criteria and on the natural history of *CDKL5*-associated encephalopathy. We screened the entire coding region of *CDKL5* for mutations in 183 females with encephalopathy with early seizures by denaturing high liquid performance chromatography and direct sequencing, and we identified in 20 unrelated girls, 18 different mutations including 7 novel mutations. These mutations were identified in eight patients with encephalopathy with RTT-like features, five with infantile spasms and seven with encephalopathy with refractory epilepsy. Early epilepsy with normal interictal EEG and severe hypotonia are the key clinical features in identifying patients likely to have *CDKL5* mutations. Our study also indicates that these patients clearly exhibit some RTT features such as deceleration of head growth, stereotypies and hand apraxia and that these RTT features become more evident in older and ambulatory patients. However, some RTT signs are clearly absent such as the so called RTT disease profile (period of nearly normal development followed by regression with loss of acquired fine finger skill in early childhood and characteristic intensive eye communication) and the characteristic evolution of the RTT electroencephalogram. Interestingly, in addition to the overall stereotypical symptomatology (age of onset and evolution of the disease) resulting from *CDKL5* mutations, atypical forms of *CDKL5*-related conditions have also been observed. Our data suggest that phenotypic heterogeneity does not correlate with the nature or the position of the mutations or with the pattern of X-chromosome inactivation, but most probably with the functional transcriptional and/or translational consequences of *CDKL5* mutations. In conclusion, our report show that search for mutations in *CDKL5* is indicated in girls with early onset of a severe intractable seizure disorder or infantile spasms with severe hypotonia, and in girls with RTT-like phenotype and early onset seizures, though, in our cohort, mutations in *CDKL5* account for about 10% of the girls affected by these disorders.

Keywords: *CDKL5*; *MECP2*; Rett syndrome; seizures

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Introduction

X-linked cyclin-dependent kinase-like 5 (*CDKL5*, OMIM 300203)-associated encephalopathy is a recently described X-linked disorder with a phenotype overlapping that of Rett syndrome (RTT, OMIM 312750) and X-linked infantile spasms (ISSX, OMIM 308350). RTT is a neuro-developmental disorder that predominantly affects girls and is the most common genetic cause of intellectual disability in females. Typically, RTT is characterized by a period of nearly normal development followed by regression with loss of social motor, and communication skills, combined with the occurrence of specific features, including hand stereotypies, microcephaly, autonomic disturbance or epilepsy (Bienvenu and Chelly, 2006). According to the criteria of Hagberg, classical RTT could be distinguished from atypical RTT. Atypical RTT can be divided into several subgroups (Hagberg *et al.*, 1994, 1995). One of these, known as Hanefeld variant, described female patients with atypical RTT and infantile spasms (Hanefeld, 1985). This variant was extended to include those with seizures of early onset before regression (Goutieres and Aicardi, 1986).

As for RTT, only girls are affected by *CDKL5*-related disorders, and so far, 26 females with *CDKL5* mutations have been described. Several mutations types and locations within the *CDKL5* sequence have been reported. Six missense mutations (Tao *et al.*, 2004; Evans *et al.*, 2005; Scala *et al.*, 2005; Archer *et al.*, 2006; Rosas-Vargas *et al.*, 2008), two nonsense mutations (Archer *et al.*, 2006; Nectoux *et al.*, 2006), six splice mutations (Weaving *et al.*, 2004; Evans *et al.*, 2005; Archer *et al.*, 2006) and eleven deletions/insertions leading to frameshifts and premature truncation (Weaving *et al.*, 2004; Mari *et al.*, 2005; Scala *et al.*, 2005; Archer *et al.*, 2006) have been reported in the *CDKL5* gene. Up to now, only one recurrent mutation (p.Ala40Val) has been identified (Rosas-Vargas *et al.*, 2008). Strikingly, these patients with *CDKL5* mutations show a similar clinical course: they had seizures in the first months of life and subsequently developed recognizable RTT features. Clinical heterogeneity corresponding to *CDKL5*-related encephalopathy has also been reported. The spectrum of phenotypes includes the following: patients with some of the diagnostic criteria of RTT early onset seizure variant, patients characterized by severe encephalopathy with refractory seizures (Evans *et al.*, 2005), patients with X-linked infantile spasms (Kalscheuer *et al.*, 2003; Evans *et al.*, 2005) and finally patients with autistic features (reduced social interaction with poor eye fixation and avoidance of eye contact and stereotypies) (Weaving *et al.*, 2004; Tao *et al.*, 2004; Evans *et al.*, 2005; Scala *et al.*, 2005; Nectoux *et al.*, 2006). However, despite these progresses, age-related key clinical symptoms and the natural history of *CDKL5*-associated encephalopathy have yet to be clearly defined.

The purpose of this paper is to help to delineate more thoroughly the natural history of *CDKL5*-associated encephalopathy and to identify the main clinical characteristics of this disorder. In addition, we aim to ascertain the frequency of mutations in *CDKL5* in the different subgroups previously identified with *CDKL5* mutations and better define diagnostic criteria that should orient molecular investigations towards *CDKL5* testing. To this end, we screened the whole coding region of the *CDKL5* gene in a total of 183 patients with encephalopathy and describe here 20 patients with pathogenic *CDKL5* mutations including 7 novel sequence variations and review the clinical features of the patients published to date.

Materials and Methods

Patients ascertainment and determination of phenotypes

A group of 183 unrelated females with encephalopathy with early seizures were referred by various Paediatric Neurology centres to our diagnostic laboratory at Cochin Hospital in Paris for *CDKL5* analysis. The group included 99 individuals with severe encephalopathy (impairment of both motor functions and communication abilities) and refractory epilepsy (EE) without infantile spasms (IS) and no RTT-like features, 30 patients with unexplained IS and 54 patients with encephalopathy with controlled epilepsy but with prominent RTT-like phenotype (deceleration of head growth, stereotypies, autonomic features and hand apraxia). All patients correspond to sporadic non-familial cases. All blood samples were obtained after receiving informed consent. For all patients, *MECP2* mutations and large molecular rearrangements in the *MECP2* locus were excluded by denaturing liquid high performance chromatography, direct sequencing and *MECP2* multiplex ligation-dependent probe amplification kit (MRC Holland) analysis. Conventional cytogenetic investigations were also normal in all patients.

Retrospective clinical history and comprehensive neurological examination data were either collected by the principal investigator (NBB, for nine patients, case numbers 1, 2, 4, 7, 9, 10, 15, 17, 19) (Tables 1 and 2) or by physicians ensuring the follow-up of the patients included in this cohort.

We paid specific attention to the RTT criteria defined by Hagberg *et al.* (1994), including the primary criteria, loss of acquired fine motor skills in early childhood, loss of acquired language, hand stereotypies, early deviant communicative behaviour, deceleration of head growth of 2 SD, and the RTT disease profile defined as a period of normal development followed by a regression, loss of acquired fine finger skill in early childhood, the characteristic intensive eye communication and the characteristic RTT electroencephalographic development (Hagne *et al.*, 1989). In addition, we also examined the prevalence of some RTT-supportive manifestations (such as breathing irregularities, bloating/air swallowing, teeth grinding, gait dyspraxia, small blue and cold impaired feet, characteristic RTT electroencephalographic features, unprompted sudden laughing/screaming spells and eye communication).

In patients with epilepsy, we paid a specific attention to the characterization of epilepsy in terms of age at onset of seizures, type and severity of seizures, and ictal and interictal EEG abnormalities. Epileptic seizures were classified according to the recommendations of the Commission on Classification and Terminology of the International League Against Epilepsy (1989).

Table 1 *CDKL5* mutations identified in this study

N° case	Initial concerns	Nucleotide change	Amino acid change	Location	Domain
Nonsense mutations					
19	AtyRTT	c.352C>T	p.Q118X	Exon 6	Catalytic
9	EE	c.425T>A	p.L142X	Exon 7	Ser/Thr kinase
20	AtyRTT	c.2500C>T	p.Q834X	Exon 18	C-Ter
Frameshift mutations					
13	EE	c.229delGAAG	p.E77HfsX111	Exon 5	Catalytic
15	ISSX	c.800_801delAT	p.N267KfsX271	Exon 10	Catalytic
10	EE	c.865insA	p.E289EfsX325	Exon 11	Catalytic
3	AtyRTT	c.1311dupC	p.S438QfsX471	Exon 12	C-Ter
2	AtyRTT	c.1892_1893dupTA	p.I631IfsX632	Exon 12	C-Ter
8	AtyRTT	c.2045_2046delAGins18	p.E682GfsX693	Exon 13	C-Ter
11	ISSX	c.2016delC	p.T672TfsX783	Exon 13	C-Ter
1	ISSX	c.2323_2324delGA	p.E775EfsX799	Exon 16	C-Ter
12	EE	c.2635_2636delCT	p.L879EfsX908	Exon 18	C-Ter
17	EE	c.2635_ 2636delCT	p.L879EfsX908	Exon 18	C-Ter
Splice mutations					
5	EE	c.64+2delT	–	Intron 2	–
4	EE	c.99+1G>T	p.G22X25	Intron 3	–
6	AtyRTT	c.145+2T>C	–	Intron 4	–
Missense mutations					
16	AtyRTT	c.119C>T	p.A40V	Exon 4	Catalytic
18	EE	c.119C>T	p.A40V	Exon 4	Catalytic
7	ISSX	c.659T>C	p.L220P	Exon 9	C-Ter
14	ISSX	c.2152G>A	p.V718M	Exon 14	C-Ter

Magnetic resonance imaging studies

Magnetic resonance imaging (MRI) of the brain was performed in all patients and systematically re-evaluated by two investigators (NBB and NB). Because MRI studies were performed at different institutions over a 10-year period, the imaging sequences varied substantially, although all patients had sagital, coronal and axial T_1-weighted as well as axial T_2-weighted studies. In addition, 10 patients had axial FLAIR studies.

Molecular methods

DNA was extracted from peripheral blood using standard methods. DNA samples were screened for mutations in *CDKL5* using transgenomic WAVE denaturing high performance liquid chromatography. The *CDKL5* coding sequences (exons 2–21; the start codon is located in exon 2) and neighbouring intronic regions were entirely analysed using primers and conditions as previously described (Scala *et al.*, 2005). PCR products resulting in abnormal denaturing high performance liquid chromatography profiles were sequenced on both strands using PCR primers with fluorescent dye terminators on an ABI 3100-avant genetic analyser (Applied Biosystems, Foster city, USA).

X-inactivation studies were performed as previously described (Allen *et al.*, 1992).

mRNA analysis

Total RNA isolation from lymphoblastoid cells or fibroblasts from *CDKL5* mutation patients, and cDNA synthesis was performed according to standard protocols. *CDKL5* mRNA was amplified by RT–PCR as five overlapping fragments spanning exons 1–6, exons 2–8, exons 7–11, exons 10–13 and exons 12–17. RT–PCR products were subsequently analysed by electrophoresis on 2% agarose gel and sequenced on both strands using Big Dye terminators-1 kit and ABI 3100-avant-equipment (Applied Biosystems, Foster city, USA). Size of amplified fragments, exonic position and sequences of primers used for RT–PCR amplification, as well as PCR conditions, are indicated in Table 1.

Plasmid construction

pEGFP-hCDKL5 containing the entire cDNA of human *CDKL5* (1030 amino acids) was kindly provided by Charlotte Kilstrup-Nielsen (Bertani *et al.*, 2006). The c.800_801delAT, c.1311dupC and c.2635_2636delCT *CDKL5* mutations were generated by site-directed mutagenesis using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to manufacturer instructions. Oligonucleotides containing each of both frameshift mutations that were designed for mutagenesis are shown in Table 2. All PCR-generated constructs were verified by sequencing.

Cell culture and transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum at 37°C with 5% CO_2. One night before transfection, cells were seeded onto round coverslips. Transient transfection experiments were performed with FuGENE6 transfection Reagent (Roche) according to manufacturer instructions. Forty-eight hours post-transfection, cells were fixed with 4% paraformaldehyde for 20 min, mounted with Vectashield mounting medium with DAPI (Vector Laboratories, ABCYS, Paris, France) and analysed with a Leica DMRA2 fluorescence microscope. To confirm the immunocytochemistry results, Western blot analysis was performed on cell fractionation protein extracts using a monoclonal anti-GFP

Table 2 Overview of the clinical features of the 20 patients with *CDKL5* mutations

Patient	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Initial concern	ISSX	AtyRTT	AtyRTT	EE	EE	AtyRTT	ISSX	AtyRTT	EE	EE	ISSX	EE	EE	ISSX	ISSX	AtyRTT	EE	EE	AtyRTT	AtyRTT
Age at last evaluation	5 yrs	19 yrs	5 yrs	4 yrs	1.5 yrs	5.5 yrs	3 yrs	2.7 yrs	17 yrs	10 yrs	2.7 yrs	2 yrs	26 yrs	1.8 yrs	4 yrs	3 yrs	10 yrs	6 yrs	8 yrs	4 yrs
Deceleration of head growth[a]	–	+	+	+	+	+	+	+	+	–	–	–	+	+	–	–	–	–	+	–
Absolute microcephaly	–	–	–	–	–	+	+	–	+	–	–	–	+	+	–	–	–	–	+	–
Regression	–	–	–	–	–	–	–	–	+	–	–	+	–	–	–	+	–	–	–	–
Severe intellectual disability	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Autistic features[b]	+	+	+	+	–	+	+	+	+	+	–	+	+	–	+	+	+	+	+	+
Walk with aid or unaided	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–	+	+	+	+	+
Limited hand skills	+	–	+	+	+	+	+	+	+	0	+	+	+	+	+	–	–	–	+	+
Hand stereotypies/onset	+	+	+	+	+	+	+	–	+	+	–	+	+	+	+	–	+	+	+	+
(yrs)	(2.5)	(3)	(3)	(3)	(1.3)	(2.5)	(2.3)		(3)	(3)		(2)	(3)	(1.5)	(2.5)		(2.5)	(3)	(2.8)	(2)
Bruxism	+	–	+	–	+	–	–	+	–	–	+	–	+	–	+	–	–	+	–	–
Sleep disturbances	+	+	+	–	+	+	–	+	+	–	+	–	+	–	+	–	–	–	+	–
Autonomic features[c]	–	–	–	–	+	–	–	–	–	+	–	–	+	–	–	–	–	–	–	–
Seizure onset (weeks)	3	6	2	8	1	10	4	10	5	5	8	5	12	3	1	4	2	6	3	1.5
Infantile spasms/onset	+	–	+	+	+	–	+	–	+	+	+	+	0	+	+	–	+	+	+	–
(mo)	(20)		(9)	(10)	(12)		(6)		(6)	(5)	(5)	(5)		(8.5)	(12)		(36)	(15)	(9)	
Late Refractory epilepsy	–	–	+	+	–	+	–	–	–	–	+	–	+	+	+	+	–	+	–	–

Initial diagnosis refers to the first diagnosis for which the *CDKL5* mutation screening was performed. EE = Encephalopathy with epilepsy; ISSX = infantile spasms; AtyRTT = atypical RTT syndrome; yrs = years; mo = months; w = weeks, (+) presence of the symptom; (–) absence of the symptom.
[a]deceleration of head growth that crosses percentiles but not necessarily reach the criteria for absolute microcephaly;
[b]autistic features includes avoidance of eye gaze, reduced social interaction;
[c]autonomic features includes breathing dysrhythmia, abdominal distension or gastrointestinal dysfunction, small narrow and cold extremities.

antibody according to manufacturer's instructions (Roche Applied Science, Indianapolis, IN, USA) as previously described (Rosas-Vargas *et al.*, 2008).

Results

Phenotypic analyses of *CDKL5*-associated encephalopathy

Of the 183 individuals studied, 20 unrelated patients presented a pathogenic *CDKL5* mutation. The 20 patients with *CDKL5* mutations were followed for an average duration of 4.5 years (range 1.5–26 years). These patients' clinical data are shown in Table 2. All patients were born at full term following an uneventful delivery. Birth head circumference was within the normal range for gestational age in all cases (mean 34.8 ± 1.33 cm). Perinatal period was normal in all cases.

Onset of symptoms

For all patients, medical attention was first sought for epileptic seizures, starting within the first 3 months of life (mean 4.97 ± 3.2 weeks), although motor developmental milestones were also severely delayed in all cases. At the age of seizures onset, all patients presented severe hypotonia (lack of head control at the first evaluation), combined with delayed visual behaviour but with no pyramidal signs.

None of the patients had a period of apparent normal development. Seventeen patients made progress although remained significantly delayed and three (15.8%, Patients 9, 12, 16) showed regression after 6 months that predominates on visual contact and babbling. Strikingly, this regression was not in association with the epileptic encephalopathy condition.

Clinical phenotype

At the age of the last evaluation (median 4.5 years, range 1.5–years), all patients presented severe developmental delay. However in all cases, it was characterized by failure to ever develop appropriate motor skills, without any loss of motor skills. Mean OFC was within normal range (mean 21st percentile). Eleven patients (55%) showed a deceleration of head growth that crosses percentiles, with 6/11 having absolute microcephaly (<3rd percentile for head circumference). Considering their best motor skills, all patients developed head control, and 13 (65%) were able to sit without support. Motor dyspraxia was evident in all cases leading to impaired ambulation, with only 6 patients able to walk with support and 13 patients unable to walk at all. Only one patient (patient 2), who is 19 years old, exhibited a milder phenotype. She was able to walk unaided although with a broad-based gate. She was able to feed herself with a spoon, and to formulate short three-word-sentences. Although we did not detect a somatic mosaicism or an X-inactivation mosaicism in blood, this milder phenotype would suggest that the majority of normal X chromosomes may remain active in the brain of this patient.

Limited hand skills were present in all cases. While in the vast majority (75%), hand use was limited to gross manipulation (i.e. touching and grasping), only five were able to transfer one object from one hand to the other, without purposeful hand use.

Neurological examination showed marked trunk hypotonia in all cases. Only 26.3% had pyramidal signs with brisk tendon reflexes and bilateral Babinski sign, but no spasticity.

Expressive language was impaired in all cases and limited to babbling or few isolated words in five patients, characterized by sporadic and echolalic words consisting of repetitions of adult utterances.

With regard to the characteristics of the epilepsy, all patients presented with early epileptic seizures. They consisted of generalized tonic seizures with flushing of the face (18 cases) and tonic followed by a clonic phase in two others. Seizures were brief, lasting <1 min but very frequent (>5 per day). No myoclonic seizures were reported at onset. At onset, interictal EEG was normal in 15 cases, and demonstrated slow background activity with expected developmental features in five cases.

Subsequently, six patients became transiently or definitively seizure free with antiepileptic drugs, and 14 others (70%) developed epileptic encephalopathy with infantile spasms at the mean age of 11.3 months (±8.3). At the age of evaluation, 15 patients had ongoing seizures, with refractory epilepsy in nine and partially controlled seizures in six cases. For the nine patients with refractory seizures, interictal EEG showed high amplitude delta waves with pseudo-periodic bursts of high amplitude spikes, polyspikes and spikes and waves that were generalized (three cases) or predominated in central (three cases), temporal (one case) or temporo-occipital (two cases) regions. Neither antiepileptic treatment nor ketogenic diet caused the slightest reduction of seizure frequency. For the six patients with partially controlled seizures (seizure frequency between one per month to one per year), EEG still showed paroxysmal activity with focal or generalized spike and wave discharges in two cases, and a slow background rhythm without spikes in two cases, and normal in two cases. Antiepileptic drugs were topiramate (one case), valproate (two cases) and vigabatrin (three cases). For the six patients without seizures, EEG was either normal in three cases or showed slow background activity with poverty of the usual rhythmic activities (three cases).

Autistic-like features were present in 17 cases (85%) with reduced social interaction and poor eye fixation. Avoidance of eye contact was present in 15 cases (75%). As there are many patients with severe to profound intellectual disability who have good eye contact, poor eye fixation should be attributed to the autistic features rather than the low cognitive level. However, although autistic features can explain poor eye contact, this can also be a consequence of visual impairment or the epileptic encephalopathy.

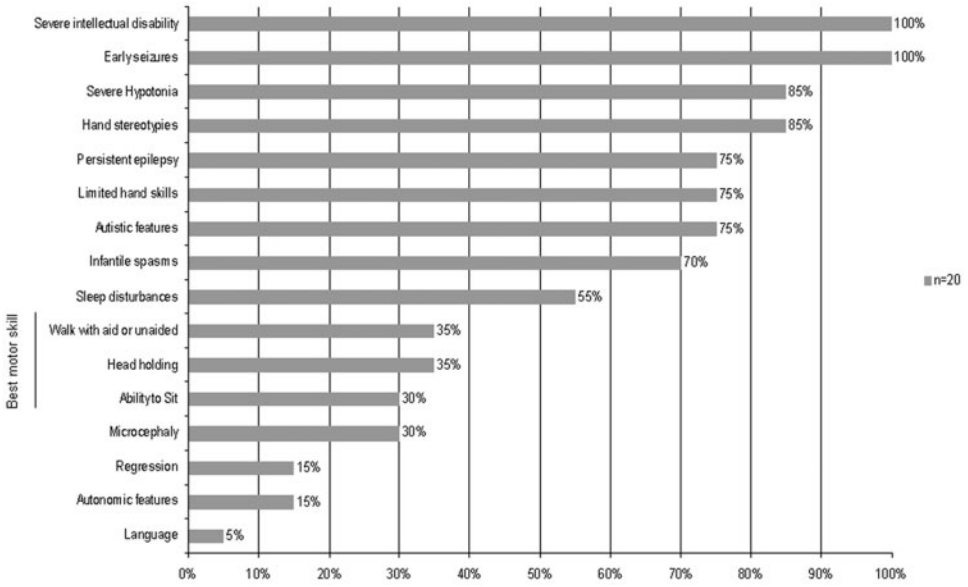

Fig. 1 Prevalence of 18 clinical features in 20 *CDKL5* mutation patients. Early seizures were observed in all mutation *CDKL5* patients.

Hand stereotypies were present in 17 cases (85%). The median age of onset of hand stereotypies was 2.5 years (range 1.3–3 years). Most stereotypies were bilateral. The most frequent hand movement observed was hand mouthing in 11 cases (55%), but also hand clapping in nine cases (45%) and hand wringing in six cases (30%). However, neither hand washing nor hair pulling stereotypy was observed. The second most frequent stereotypies observed was bruxism (eight cases, 40%). Trunk rocking and cervical retropulsion was reported in five cases (25%). Strikingly, most patients (75%) had more than one stereotypy with bruxism. Only one patient (Patient 16) had neither bruxism nor stereotypies, and two (Patients 8 and 11) had isolated bruxism.

Breathing dysrhythmia was present in three cases (Patients 5, 10, 13), which consisted of intermittent and occasional episodes of hyperventilation. They were never associated with breath holding or forced breathing or any sign of abdominal distension. Small and narrow cold feet were observed in three cases. Other dysautonomic features such as flushed face and dilated pupils were absent.

Sleep disturbances were reported in 11 cases (55%) that consisted in night awaking with screaming spells. Only one patient (Patient 2) had occasional aggressive outburst.

In no case, did we observe the typical RTT syndrome disease profile with a period of normal development, followed by a period of regression with subsequent limited recovery of social communication skills.

Figure 1 shows the frequency of each of the clinical features in *CDKL5*-associated encephalopathy. We tried to compare the group of 'the most severe *CDKL5* encephalopathy' ($n = 13$) versus 'the less severe *CDKL5* encephalopathy' ($n = 7$) (Table 3). Although not statistically significant, the main difference between the two groups is their ambulatory abilities. Some additional differences are also observed affecting deceleration of head growth, microcephaly, severity of hand apraxia, bruxism and sleep disturbance. However, there was no difference for incidence of refractory epilepsy, hand stereotypies or poverty of eye gaze. Moreover, considering brain MRI (see below), the most severe group showed a higher prevalence of posterior white matter hyperintensity on T2/FLAIR sequence (8/13 versus 1/7), although their median age was younger than the less severe *CDKL5* mutation patients (3.25 years compared with 7.5 years in the less severe group).

Brain MRI

Brain MRI was performed in all *CDKL5*-mutated cases. The median age at the last brain MRI was 4 years (range 1.8–17 years). Cortical atrophy was observed in 13 cases (65%), and its association with mild cerebellar atrophy was present in two cases (Fig. 2A). Areas of high signal were seen in the posterior white matter on T2/FLAIR in nine cases (45%) associated with hyperintensities in the dentate nuclei in three cases (15%) (Fig. 2B). Marked white matter hyperintensities in both temporal lobes on T2/FLAIR sequences was also noticed in six cases out of nine cases at an age when one would expect the brain to be fully myelinated (age of >3 years on MRI) (Fig. 2C). In four cases (20%), brain MRI was considered normal. Neither cortical malformations nor dysplasia were observed. Spectroscopy and diffusion-weighted imaging (DWI) sequences were also considered normal.

CDKL5 mutational analysis

Eighteen different mutations were identified in 20 unrelated patients (Table 1). Six of the mutations have been previously reported (c.800_801delAT, c.1311dupC,

Table 3 Comparison of clinical features in most severe forms ($n = 13$) and less severe forms ($n = 7$) of *CDKL5* mutation patients

	More severe *CDKL5* encephalopathy ($n = 13$)		Less severe *CDKL5* encephalopathy ($n = 7$)	
Age at last evaluation (years)	6.80 ± 7.66		8.57 ± 5.35	
Walk with aid or unaided	0	0.0%	7	100.0%
Microcephaly (OFC < 3rd *P*)	5	38.5%	1	+4.3%
Regression	2	15.4%	1	14.3%
Severe intellectual disability	13	100.0%	7	100%
Autistic features	10	76.9%	7	100.0%
Language	0	0.0%	1	14.3%
Hypotonia	13	100.0%	4	57.1%
Best motor skills				
Head holding	7	53.8%	0	0.0%
Ability to Sit	6	46.2%	0	0.0%
Limited hand skills	13	100.0%	2	28.6%
Hand stereotypies	11	84.6%	6	85.7%
Combination of hand stereotypies	9	69.2%	6	85.7%
Hand clapping	6	46.2%	3	42.9%
Hand mouthing	5	38.5%	6	85.7%
Hand wringing	4	30.8%	2	28.6%
Bruxism	7	53.8%	1	14.3%
Sleep disturbances	9	69.2%	2	28.6%
Hyperventilation	3	23.1%	0	0.0%
Autonomic features	2	15.4%	1	14.3%
Seizure onset (weeks)	4.91 ± 3.65		4.33 ± 1.63	
Infantile spasms/epileptic encephalopathy	10	76.9%	4	57.1%
Onset (months)	9.83	4.58	16.25	13.79
Ongoing seizures	11	84.6%	4	57.1%
Refractory epilepsy	6	46.2%	3	42.9%
Brain MRI				
Median age	3.25 yrs	Range 1.8–15 yrs	7.5 yrs	Range 2–17 yrs
Normal	1	7.7%	3	42.9%
Cerebral atrophy	9	69.2%	4	57.1%
Abnormal myelination in the temporal lobes	3	23.1%	3	42.9%
Posterior white matter hyperintensity on T2/FLAIR	8	61.5%	1	14.3%
Hyperintensity on dentate nuclei	3	23.1%	0	0.0%
Cerebellar atrophy	1	7.7%	1	14.3%

The main difference between the two groups are their ambulatory abilities, though non-statistically significant. Most severe *CDKL5* mutations patients (i.e. non-ambulatory) also show more frequent microcephaly, severity of hand apraxia, of bruxism, sleep disturbances and posterior white matter hyperintensity on T2/FLAIR sequence.

c.2635_2636delCT, c.99+1G>T, p.Ala40Val and p.Leu220Pro) (Bahi-Buisson *et al.*, 2008; Rosas-Vargas *et al.*, 2008). Three of them were nonsense mutations, nine were frameshift mutations, three were splice mutations and three were missense mutations. Mutations were located in exons 4–7, 9–14, 16 and 18 and in introns 2–4 of the *CDKL5* gene (Fig. 3). None of the mutations was detected in the parents and in 100 control individuals of different ethnic origins. In all studied cases, no X-chromosome inactivation bias was detected (Table 1).

CDKL5 is a large protein of 1030 amino acids with a conserved serine–threonine kinase domain within its N-terminus and a large C-terminal region involved in either the catalytic activity or the subcellular localization. The three nonsense mutations generate a stop codon at positions 118, 142 and 834, and the nine frameshift mutations generate a stop codon at positions 111, 272, 325, 462, 632, 693, 783, 799 and 908 and would expect to produce truncating CDKL5 protein lacking a large region of the C-terminal region. Size analysis and direct sequencing of RT–PCR products were performed to study the transcriptional consequences of these mutations for patients with *CDKL5* mutations for which lymphoblastoïd or fibroblast cells were available. For instance, we found that only the expected normal fragment amplified from the normal transcript was present in fibroblast cells from the patient carrying the c.1892_1893dupTA mutation (Fig. 4), and that the expression level of the mutated transcript was very significantly reduced in lymphoblastoïd cells from the patient carrying the p.Leu142X mutation (Fig. 4). These results suggest that these mutated transcripts are likely to be highly unstable, due to mRNA surveillance and

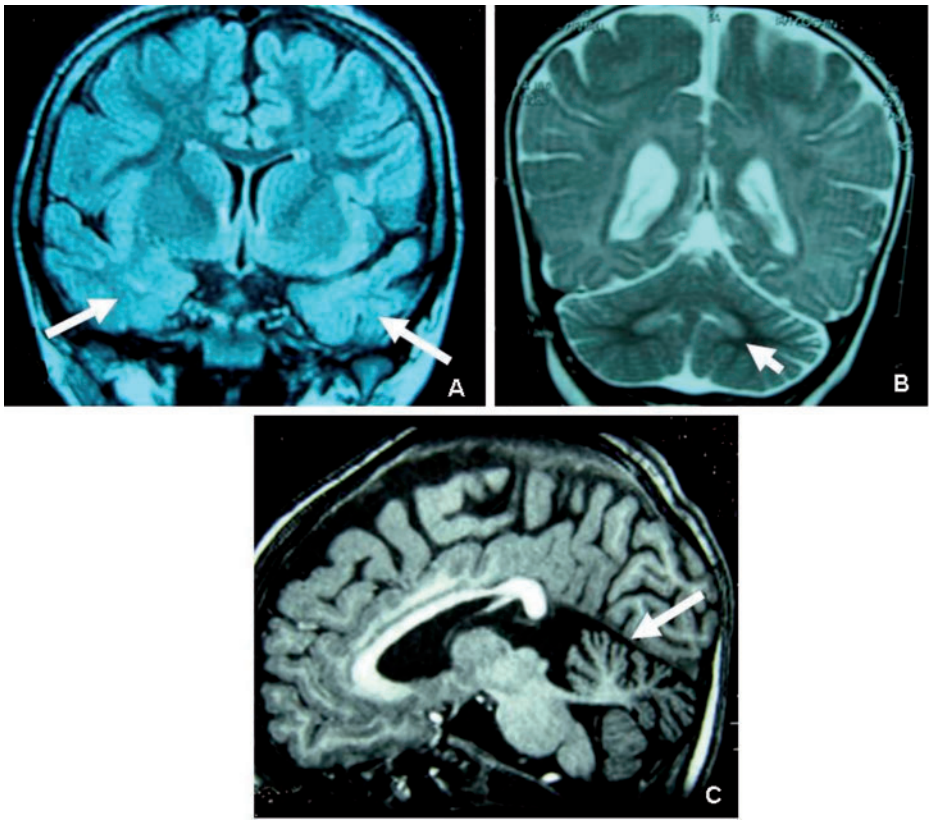

Fig. 2 Brain MRI findings for patients with *CDKL5* mutations. Abnormalities consistently noticed include (**A**) marked white matter hyperintensities in the temporal poles (T2 FLAIR sequence), (**B**) moderated hyperintensities of the dentate nuclei (T2 FLAIR), (**C**) cerebellar atrophy (T_1-weighted sagittal images). Arrowheads show the location of the main abnormalities in each figure.

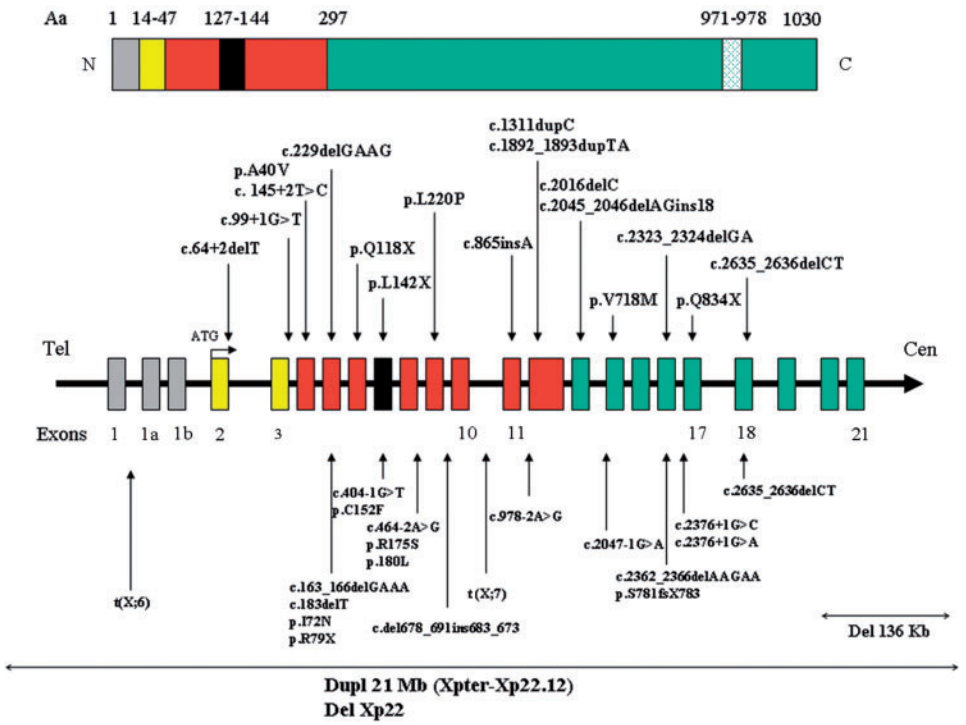

Fig. 3 Schematic representation of the *CDKL5* gene. *CDKL5* exons are indicated by boxes. Mutations that have been reported to date are given below the diagram, and those described in the manuscript are indicated above the diagram. Known functional domains are indicate: 14–47 ATP-binding site [amino acids (aa) 14–47]; Ser–Thr protein kinase active site (aa 127–144); signal peptidase serine active site GTSMCPTL (aa 971–978).

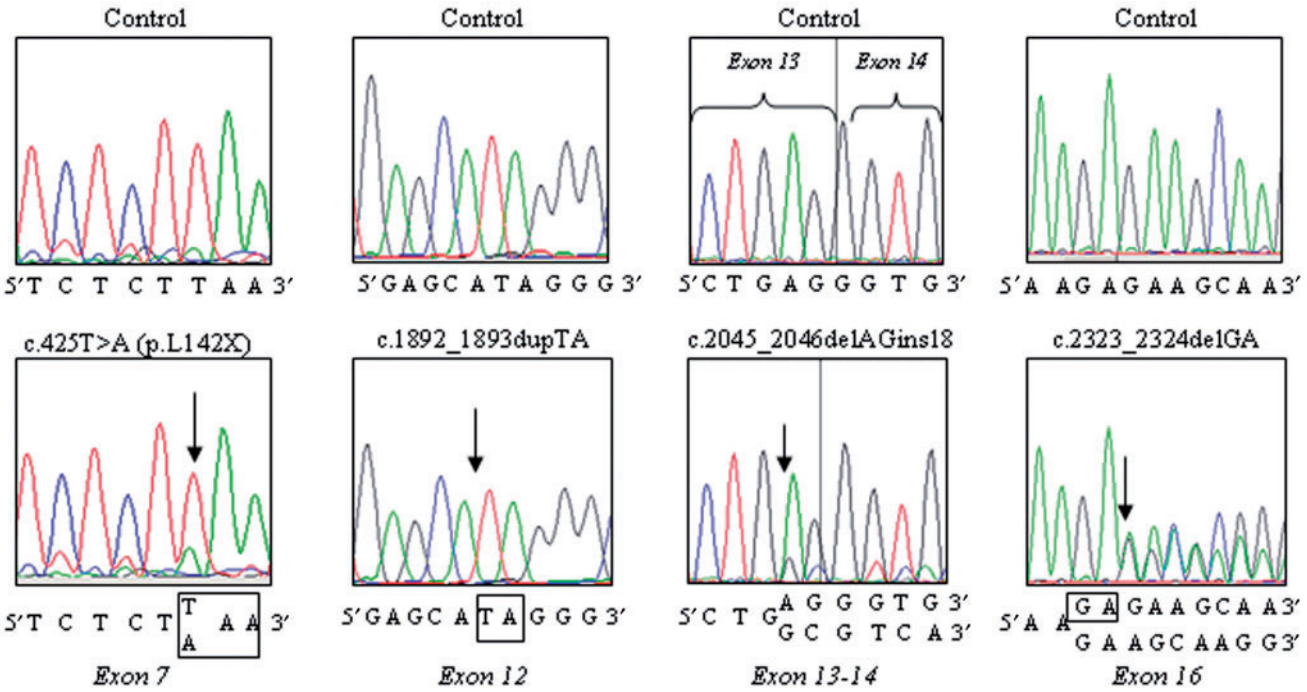

Fig. 4 RT–PCR amplification from lymphoblastoid or fibroblast cells-derived mRNA from *CDKL5* mutation patients. Fluorescence sequence analysis of RT–PCR products of the *CDKL5* gene using the forward primers. The arrows indicate the position of the *CDKL5* mutations (p.Leu142X in exon 7, c.1892_1893dupTA in exon 12; c.2045_2046delAGins18 in exon 13; c.2323_2324delGA in exon 16).

nonsense-mediated mRNA decay (NMD) phenomenon (Hentze and Kulozik, 1999; Wagner and Lyke-Andersen, 2002).

However, for two other *CDKL5* mutations (c.2323_2324delGA and c.2045_2046delAGins18), both normal and mutated alleles were expressed (Fig. 4). It is worth noticing that these two latter mutations are more distal than the previous ones and therefore probably not subjected to the NMD process. As mutated alleles from patients bearing frameshift mutations are expressed in some cases (such as c.2323_2324delGA and c.2045_2046delAGins18), and to better understand the pathogenic effect of different frameshift mutations, we compared the subcellular distribution of three previously reported mutations (c.800_801delAT, c.1311dupC, and c.2635_2636delCT; Bahi-Buisson *et al.*, 2008) by over-expression of the *CDKL5* cDNA corresponding to these mutations fused to GFP in COS7 cells. Figure 5 shows a representative localization pattern of each of the CDKL5 mutants, where the cell nucleus was identified with DAPI staining. Wild-type CDKL5 was predominantly nuclear, but was also present in the cytoplasm (Fig. 5A). Prediction of nuclear localization signals using Wolfsort program (http://wolfsort.org) suggested the presence of two putative Nuclear Localization Signal (NLS) at position 312 (RSAKRKPYH) and at position 784 (RSMKKKKKKSQ). The most proximal truncating mutation (c.800_801delAT), predicted to result in a protein lacking 759 amino acids, including the two putative NLS of the C-terminal part of the CDKL5 protein, had completely lost the nuclear staining and localized in the cytosol (Fig. 5A). The more distal truncating mutation (c.2635_2636delCT), predicted to result in a protein lacking 123 amino acids of the C-terminal part of the CDKL5 protein, had also lost the nucleus staining, but the fusion protein appeared to be localized in the perinuclear space. This observation reinforces the fact that a C-terminal region of CDKL5 is necessary for its subnuclear localization via probably protein–protein interactions (Bertani *et al.*, 2006). However, the c.1311dupC mutation predicted to result in a deletion of 569 amino acids from the C-terminal part of the CDKL5 protein did not alter the subcellular localization and the fusion protein was detected only in the nucleus. Interestingly, the c.1311dupC mutation produces a premature stop codon after addition of 24 new amino acids downstream to the mutation (Fig. 5B). Prediction of subcellular localization using LOCtree (http://cubic.bioc.columbia.edu/services/loctree) suggested that this new peptide has a nuclear localization, and could explain the specific subcellular localization of this mutant. Prediction using another program named LOCSVMpsi (http://bioinformatics.ustc.edu.cn) also suggests that this truncated protein has a nuclear localization (expected accuracy 79%). In contrast, this program predicted that the c.800_801delAT mutant has a cytoplasmic localization (expected accuracy 89%) in agreement with our immunofluorescence data (Fig. 5A).

In this study, we also reported three splice site mutations, two of which are novel mutations (c.64+2delT and

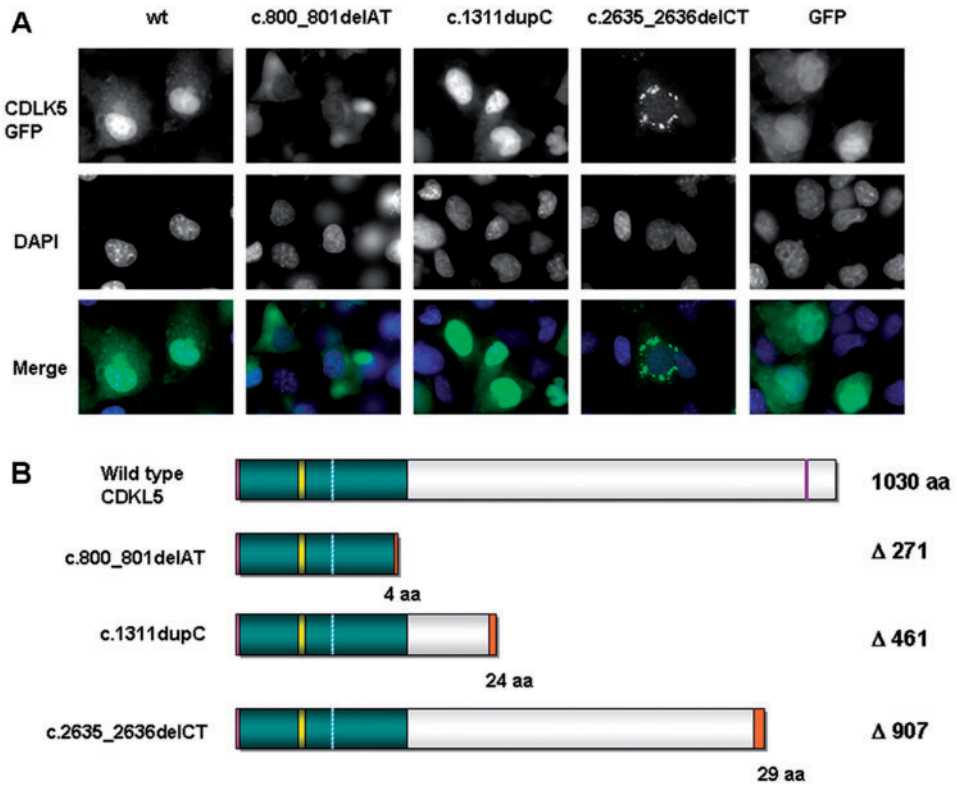

Fig. 5 Cellular localization of c.800.801delAT, c.1311dupC and c.2635.2636delCT mutated CDKL5. GFP-CDKL5 fusion proteins were overexpressed on to COS7 cells. Fixed cells were mounted in Vectashield mounting medium with DAPI. (**A**) Upper row shows GFP expressing cells, the middle row shows DAPI nuclear staining and the lower row shows the merge of both the upper and the middle rows with GFP in green and nuclei stained in blue. (**B**) Putative translational consequences of the three truncated mutations.

c.145+2T>C), identified in three unrelated affected girls. All disrupt the GT dinucleotide of the splice donor site. To test the consequences of these mutations on mRNA expression, we performed RT-PCR using RNA extracted from cultured lymphoblastoïd cells (for the c.99+1G>T mutation) or fibroblasts (for the c.64 + 2delT and c.145+2T>C mutations) of the *CDKL5* mutation patients. Amplification of the cDNA fragment encompassing exons 2–8 using fibroblasts' RNA of the patient's bearing the c.99+1G>T mutation produced two different RT–PCR products. Sequencing of the smaller RT–PCR products showed that exon 3 was skipped, which would result in a frameshift and premature stop codon three amino acids downstream to the one in position 22 (p.G22X25) and then premature truncation of the protein (Fig. 6). However, no abnormal transcripts were detected in the two other patients suggesting that the mutated transcripts were probably unstable (Fig. 6).

Four patients were identified with *CDKL5* missense mutations: c.119C>T/p.Ala40Val in two unrelated patients, c.659T>C/p.Leu220Pro and c.2152 G>A/p.Val718Met (Fig. 7). For three of these patients, molecular data have recently been reported (Rosas-Vargas *et al.*, 2008). However, for the purpose of this study, these patients were re-visited and their clinical and imaging data were compiled. The additional p.Val718Met missense mutation is located in the C-terminal region of CDKL5 and changes a residue that is highly conserved during evolution (Fig. 7).

Finally, in addition to these pathogenic mutations detected in a total of 20 patients, the screening of *CDKL5* exons and their flanking intronic sequences led to the identification in intron 8 of a frequent sequence variation, c.555-19C > G, in six unrelated patients (three EE, one ISSX, two RTT-like). This sequence variation has been previously found by Archer and colleagues in patients with severe mental retardation and seizure onset in the first year of life, or with infantile spasms of unknown aetiology, and has been classified as a mutation of uncertain significances (Archer *et al.*, 2006). In our study, we showed that asymptomatic mothers of two patients bearing this nucleotide substitution with a non-biased X-chromosome inactivation profile presented the same sequence variation, which rules out the involvement of this nucleotide substitution in the aetiology of the phenotype.

The overall frequency of mutations in the cohort of 183 selected patients with encephalopathy and early seizures referred for *CDKL5* screening represents ~11%. However, if we take into consideration the clinical description and the classification of the patients in three groups (i) encephalopathy with RTT-like features ($n = 54$), (ii) infantile spasms ($n = 30$) and (iii) encephalopathy with refractory epilepsy ($n = 99$), the following distribution of mutations frequencies

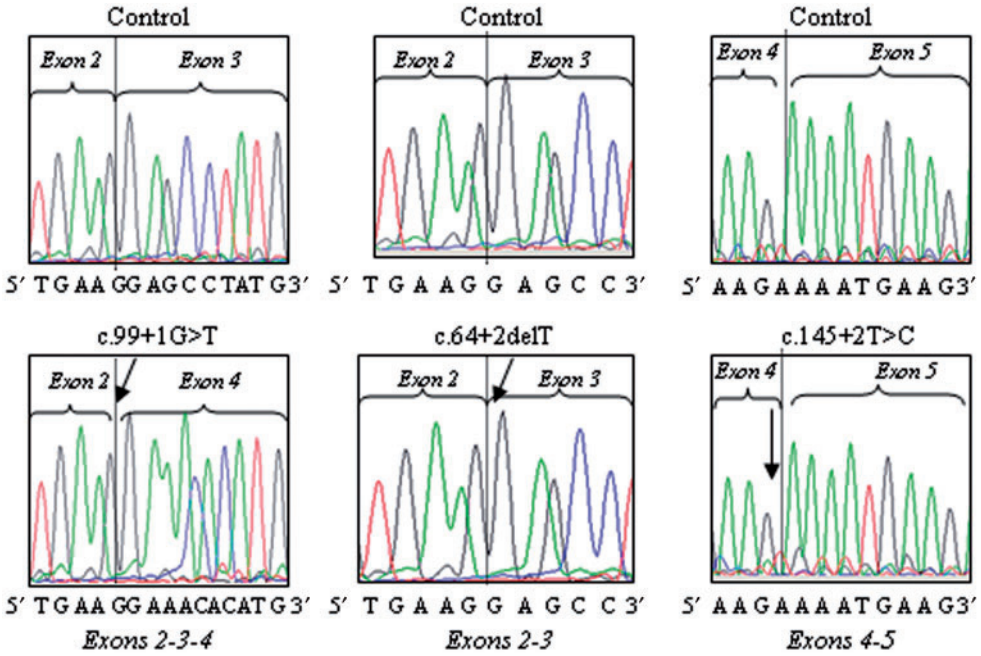

Fig. 6 RT–PCR amplification from lymphoblastoïd or fibroblast cells-derived mRNA from *CDKL5* mutation patients. Fluorescence sequence analysis of RT–PCR products of the *CDKL5* gene using the forward primers. The arrows indicate the position of the mutated nucleotide (c.64+2delT in intron 2; c.99+1G>T in intron 3; c.145+2T>C in intron 4).

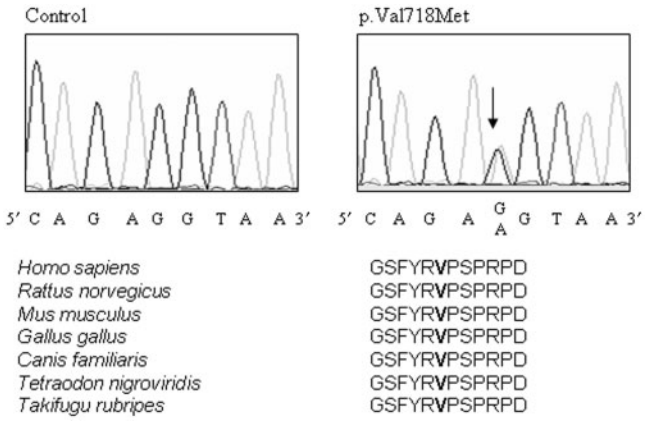

Fig. 7 Fluorescence sequence analysis of exon 14 of the *CDKL5* gene. The arrow indicates the position of the mutation p.Val718Met. Multiple alignment of a part of the C-terminal region of CDKL5 protein.

could be depicted. Eighteen different mutations were identified in eight unrelated patients with encephalopathy with refractory epilepsy (8/99, 8.1%), five in patients with infantile spasms (5/30, 16.7%) and seven in patients with RTT-like phenotype (7/54, 12.9%). These differences were not statistically significant.

Discussion

This paper describes the spectrum of neurological features associated with *CDKL5* mutations in 20 girls aged from 1.5 to 26 years and aims to highlight the key clinical features of this rare encephalopathy that should help establishment of molecular diagnosis. At the age of diagnosis, eight were referred for *CDKL5* screening because of encephalopathy with epilepsy, five for infantile spasms and seven for RTT-like phenotype. Accurate analysis of their disease history and phenotypes allows us to point out the most important and distinctive characteristics of the *CDKL5*-associated disorders that combine early seizures in all cases evolving into severe encephalopathy with RTT-like features in the majority of patients and refractory epilepsy in over half of the patients. These main common features in patients with *CDKL5* mutations are shown in Fig. 1. When compared with the 24 cases previously reported in the literature (Tables 4 and 5), one of the added value of this study is the young age of the patients we present here (median age 4.5 years here versus 9 years in the literature). All together, these distinct cohorts of *CDKL5* mutation patients allow us to define some specific features of the *CDKL5*-associated encephalopathy with early features and additional typical signs that appear with age. In younger patients before 2 years of age, early onset epilepsy is probably the most consistent sign in *CDKL5* mutations. In all cases, epilepsy starts within 3 months with very frequent seizures and an interictal EEG pattern that is normal or shows background slowing. At this age, neurological examination reveals severe hypotonia and poor eye contact, but none of the RTT-like features observed in later ages. Subsequently, a large proportion of patients (70%) develop epileptic encephalopathy characterized by the occurrence of infantile spasms with hypsarrhythmia, and then multifocal epilepsy. This evolution of epilepsy into a three-step pattern in *CDKL5* mutation

Table 4 Clinical features of patients with *CDKL5* mutations, previously reported

		Clinical features										Epilepsy			
Patient/age	Mutation	Regression	Severe intellectual disability	Deceleration of head growth	Poor eye fixation	Stereotypies/Bruxism	Language	Best motor skill	Hand apraxia	Automic features	Air swallowing/Reflux	Sz onset	IS/EE	Late epilepsy	References
1/7yrs	T(X;7)(p22.3;p15)	–	+	+	+	–	–	None	NK	–	–	3–6 w	+	+	(Kalscheuer *et al*., 2003)
2/3 yrs	T(X;6)(p22.3;q14)	+	+	–	+	–	–	None	NK	–	–	NK	+	+	(Kalscheuer *et al*., 2003)
III:1/19 yrs	c.183delT	+	+	+	+	Hand mouthing wringing (1.5 yrs)	–	Crawl	+	Breathing irregularities/small feet	–	9 w	+	+	(Weaving *et al*., 2004)
III:2/19 yrs	c.183delT	–	–	–	+	–	+ echolalic	Walk	NK	–	–	NK	–	–	(Weaving *et al*., 2004)
III:3/16 yrs	c.183delT Male	–	–	+	+	–	–	None	NK	Breathing irregularities	Reflux	9 w	+	+	(Weaving *et al*., 2004)
II:1/28 yrs	IVSI3-1G>A (c.2047delG)	+	+	+	+	Hand	–	Walk	–	Breathing irregularities/small feet	–	6 w	+	+	(Weaving *et al*., 2004)
II:2/13 yrs	IVSI3-1G>A (c.2047delG)	+	+	+	–	Hand	–	Walk	+	Breathing irregularities/small feet	–	NK	–	NK	(Weaving *et al*., 2004)
II:2/5 yrs	c.455 G>T (p.CI52F)	–	–	NK	+	–	–	Head control	NK	–	–	NK	+	+	(Tao *et al*., 2004)
II: 2/41 yrs	c.525 A>T (p.CI52F)	–	+	NK	+	Hand	–	Walk	NK	Breathing irregularities	–	NK	+	+	(Tao *et al*., 2004)
II:3/41 yrs	c.525 A>T (p.RI75S)	–	+	NK	+	Hand	–	Walk	NK	Breathing irregularities	Air swallowing	NK	+	NK	(Tao *et al*., 2004)
I//7.5 yrs	IVS 7.2A A>G (p.GI55fsXI97)	–	+	+	+	Hand mouthing wringing	–	Head control	+	Cold feet	Reflux	3 w	+	+	(Evans *et al*., 2005)
2/5.5 yrs	IVSI6+1G>C (K760fsX769)	–	+	+	+	Hand wringing	–	Head lift	+	Cold feet	Reflux	8 w	+	+	(Evans *et al*., 2005)
3/11 yrs	c.215T>A (p.I72N)	–	+	+	+	Hand mouthing wringing clapping (3 yrs) Bruxism	–	Walk with support	+	Breathing irregularities/Cold feet	Reflux	4 w	–	+	(Evans *et al*., 2005)
1/7 yrs	c.838.847del10 (p.T28IfsX284)	–	+	–	+	Hand mouthing clapping	–	Sit	+	Cold feet	Reflux	5 w	+	+	(Grosso *et al*., 2006; Mari *et al*., 2005)
2/2 yrs	c.2343delG (p.S78IfsX783)	+	+	–	+	Hand mouthing clapping	Words	Walk	+	–	Reflux	3 w	–	+	(Mari *et al*., 2005)
1/9 yrs	c.163.166delGAAA (p.R55fsX74)	–	+	–	+	Hand mouthing (1 yr Bruxism)	Words	Sit	+	Breathing irregularities/Cold feet	–	1.5 mo	NK	+	(Scala *et al*., 2005)
2/8 yrs	c.2635.2636delCT (p.E879fsX908)	–	+	NK	+	Hand mouthing (4 yrs Bruxism)	Word	Sit	+	–	–	10 d	+	+	(Scala *et al*., 2005)
1/9.5 yrs	Del (Exon 18)	–	+	–	NK	NK	–	NK	+	NK	NK	2 w	–	+	(Buoni *et al*., 2006)
2/7.4 yrs	Del (c.838.847)	–	+	–	NK	NK	–	None	+	NK	NK	1 w	–	+	(Buoni *et al*., 2006)
3/9.4	Del (Exon 5)	–	+	NK	NK	NK	–	Sit	+	NK	NK	1.5 mo	–	+	(Buoni *et al*., 2006)
1/18 yrs	c.2362.2366delAAGA	–	+	–	+	+	+	Walks	+	–	NK	2 mo	+	+	(Archer *et al*., 2006)
2/2 yrs	IVS6.1 G>T	+	+	+	+	+	–	None	+	–	NK	10 d	+	+	(Archer *et al*., 2006)
3/7 yrs	IVSI1.2A>G	–	+	+	+	+	–	None	+	+	NK	3 d	NK	NK	(Archer *et al*., 2006)
4/4yrs	c. del678.691ins683.673	–	+	+	+	+	–	None	+	–	NK	1 mo	+	NK	(Archer *et al*., 2006)
5/2 yrs	IVSI6 + 1 G>A	–	+	+	–	+	–	Sit	+	+	NK	5 w	–	+	(Archer *et al*., 2006)
6/NK	c.175 C>T (p.R59X)	NK	+	NK	NK	NK	NK	Limited	NK	NK	NK	NK	NK	NK	(Archer *et al*., 2006)
7/13 yrs	c.539 C>T (p.PI80L)	–	+	+	–	+	–	Crawl	+	–	NK	6 w	NK	+	(Archer *et al*., 2006)
Total		5/23	22/24	11/17	17/20	18/21	5/23		18/19	12/20	6/14		12/20	18/19	

Yrs = years; mo = months; w = weeks; d = days; Fam = Family; RS = rett syndrome; sz = seizures; IS = infantile spams; EE = epileptic encephalopathy; NK = non-known. + = presence of the sign; – = absence of the sign. For the total, we excluded the two patients that either add unbalanced translocation (Kalscheuer *et al*., 2003) and the male patient (Weaving *et al*., 2004), -figured in grey shades in the table- in order to evaluate only patient with comparable genotype (i.e. female with *CDKL5* mutation).

Table 5 Clinical features of our patients with *CDKL5* mutations compared with other published cases

	Our series ($n = 20$)	Published cases ($n = 24$)	Combined series
	Number of cases	Number of cases	Total (Percentage)
Age at last evaluation (range)	4.5 yrs (1.5–26)	9 yrs (2–41)	
Normal pre and perinatal period	20 (100%)		
Deceleration of Head growth	11 (55%)	11/17	22 (61.1%)
Regression	3 (15%)	5/23	8 (19.0%)
Severe intellectual disability	20 (100%)	22/24	40 (93.0%)
Poor eye fixation and poursuit	15 (75%)	17/20	31 (79.5%)
Language	1 (5%)	5/23	6 (14.3%)
Hypotonia	17 (85%)	23/24	39 (90.7%)
Head holding:-virtually no motor development	7 (35%)	10/23	17 (40.5%)
Ability to sit	6 (30%)	5/23	11 (26.2%)
Walk with aid or unaided	7 (35%)	8/23	14 (33.3%)
Limited hand skills	15 (75%)	18/19	36 (94.7%)
Hand stereotypies	17 (85%)	18/21	34 (85.0%)
Hand clapping	9 (45%)	3/8	11 (40.7%)
Hand mouthing	11 (55%)	7/8	17 (63.0%)
Hand wringing	6 (30%)	8/8	14 (51.9%)
Trunk rocking	5 (25%)		5 (26.3%)
Sleep disturbances	11 (55%)	2/20	13 (33.3%)
Bruxism	8 (40%)	4/8	12 (44.4%)
Autonomic features	3 (15%)	12/20	15 (38.5%)
Air swallowing/Reflux	3 (15%)	6/14	9 (27.3%)
Early seizures	20 (100%)	18/20	37 (94.9%)
Seizure onset (wks)	5.15 ± 3.18	4.41	
Infantile spasms/epileptic encephalopathy	14 (70%)	12/20	26 (66.7%)
Onset (mo)	11.32 ± 8.3		
Persistent epilepsy	15 (75%)	18/22	33 (80.5%)

For this comparison, we excluded the male Fam 1 (III3/16 yrs, described by Weaving *et al*., 2004) and all patients that presented disrupted *CDKL5* and unbalanced translocation (Kalscheuer *et al*., 2003).

patients was described previously in 13 patients (Bahi-Buisson *et al.*, 2008) and is reinforced by this larger series.

From the age of 2–3 years, our data indicate that *CDKL5* mutations are responsible for a specific severe encephalopathy that combines in most cases epilepsy and some RTT features, which is in accordance to the small number of descriptions of the so-called Hanefeld variant of RTT (Hanefeld, 1985; Goutieres and Aicardi, 1986; Hagberg and Skjeldal, 1994). Considering epilepsy after the age of 2–3 years, 75% of *CDKL5* mutation patients had ongoing seizures, either with a partial seizure control or with refractory epilepsy, whereas 25% of *CDKL5* mutation patients became progressively seizure free. This evolution also confirms our previous findings on the three step pattern that characterizes *CDKL5* associated encephalopathy (Bahi-Buisson *et al.*, 2008). Moreover, *CDKL5* mutation patients clearly exhibit some RTT-like features such as deceleration of head growth, stereotypies and hand apraxia, and sleep disturbances (Hagberg and Skjeldal, 1994; Temudo *et al.*, 2007). Most patients (75%) show a combination of different hand stereotypies without hand gaze and bruxism, although these features become really evident in older and ambulatory patients. Conversely, dysautonomic features such as breathing disturbances and gastrointestinal dysfunction are rare, suggesting a possible better preserved autonomic system in *CDKL5* mutation patients compared to typical RTT ones.

Evaluation of motor delay in *CDKL5* mutation patients indicates that all girls are severely delayed, with very limited, if there is any, autonomy. However, according to their ambulatory ability, two groups seem to emerge. *CDKL5* mutation patients who did not acquire ambulation seem to have more severe microcephaly, hand apraxia, poverty of eye communication, bruxism and sleep disturbances. Conversely, the patients able to walk seem be have a better eye gaze, hand use and less bruxism and sleep disturbance. However, the prevalence of refractory epilepsy and hand stereotypies are comparable between the two groups.

Brain MRI is usually abnormal in *CDKL5*-associated encephalopathy. Although non-specific, we found that most patients exhibited cortical atrophy combined with hyper-intensities in the temporal lobe white matter that could be related to abnormal myelination. It is of interest to note that these features were not different between the two groups—severely affected versus less severely affected—suggesting that they constitute a marker of the disease but not a clinical indicator of prognosis.

We also highlight some negative signs in *CDKL5*-related encephalopathy currently observed in typical RTT, such as the absence of non-epileptic seizures and 'RTT disease profile'. Indeed, *CDKL5* mutation patients never show non-epileptic episodes of motor activity, such as twitching, jerking, head turning, falling forward and trembling, as

well as episodes of staring, laughing, vacant spells, and pupil dilatation frequently associated with RTT phenotype (Glaze *et al.*, 1998; Steffenburg *et al.*, 2001). Moreover, the evolution of *CDKL5*-related encephalopathy never shows a 'RTT disease profile' that consists of a period of normal development followed by a regression, loss of acquired fine finger skill in early childhood and the characteristic intensive eye communication. Finally, *CDKL5* mutation patients never demonstrated the characteristic EEG development observed in typical RTT (Hagne *et al.*, 1989). In the latter, EEG pattern evolves into different stages that correlates with clinical staging. During the stage I ('developmental arrest'), between the ages of 6–18 months, EEG pattern is slow during wakefulness and normal during sleep, without any epileptiform abnormalities. Subsequently, during the stage II ('regression') that occurs typically between the ages of 1–4 years, EEG is characterized by loss of occipital dominant rhythm and occurrence of rhythmic fronto-central theta activity and generalized spike and wave discharge during sleep initially then during wakefulness and sleep. Later, during the evolution toward the stage III ('pseudo-stationary') and IV ('late motor deterioration') EEGs, background activity becomes diffusely slower (theta to delta activity) with the occurrence of multifocal spike and sharp-waves discharges and generalized slow spike and wave activity during wakefulness and sleep (Hagne *et al.*, 1989). These features were never reported, neither in our patients with *CDKL5* mutations nor in previous reports (Archer *et al.*, 2006; Buoni *et al.*, 2006; Grosso *et al.*, 2007; Bahi-Buisson *et al.*, 2008).

Though most patients with *CDKL5* mutations exhibit encephalopathy, the clinical spectrum still remains heterogeneous and ranges from very limited developmental progress to a less severe phenotype with some language development and limited ambulation. Several hypotheses could be proposed to explain the phenotypic heterogeneity observed in female carrying a *CDKL5* mutation: (i) the nature and the location of the *CDKL5* mutation; (ii) the pattern of X-chromosome inactivation; (iii) differences in the molecular and cellular consequences of the mutations. Neither the nature of the mutation nor the X-chromosome inactivation pattern appears to correlate with the clinical heterogeneity. Missense, splicing or truncated mutations were identified in the different groups of patients and the phenotype associated with truncating mutations was variable and did not clearly correlate with the position of the mutation. Moreover, all patients in our study had a similar balanced XCI pattern in cells from peripheral blood, though we cannot exclude the fact that XCI pattern in blood leukocytes may not reflect the XCI patterns in the brain. The absence of phenotype–genotype correlation shown in this study has also been observed by other groups. For example, frameshift mutations lead to early seizure RTT variant (Mari *et al.*, 2005; Scala *et al.*, 2005) or to X-linked West syndrome (Archer *et al.*, 2006), and splicing mutations were observed in X-linked West syndrome as well as in epileptic encephalopathy (Archer *et al.*, 2006). Though further investigations are required, our results suggest that the clinical heterogeneity could be related to differences in the molecular and cellular consequences of each of the *CDKL5* mutations. For several frameshift mutations (such as c.2323_2324delGA and c.2045_2046delGC), we can detect mutated and normal transcripts suggesting that a truncated protein would be produced. In addition, our immunofluorescence data suggest that all the truncated proteins are probably not the same cellular localization. For example, the truncated protein associated with the c.1311dupC mutation was located in the nucleus and could exert a certain function, whereas the mutations c.800_801delAT and c.2635_2636delCT were associated with a mislocalization of the truncated CDKL5 proteins in the cytoplasm. Similarly, in the recent study, our group showed that missense mutations such as p.Ala40Val and p.Leu220Pro also cause mislocalization of the mutant CDKL5 protein in the cytoplasm (Rosas-Vargas *et al.*, 2008). These results suggest that these truncated proteins, if they were produced *in vivo*, may have different abnormal functional consequences. Indeed, it has been shown that a portion of the C-terminal domain is responsible for a stable residency in this cellular compartment probably through protein–protein interactions and that the C-terminal tail seems to act as a negative regulator of the catalytic activity of CDKL5 (Bertani *et al.*, 2006).

For splice mutations, qualitative and quantitative differences on mRNA maturation are well known. For instance, splice mutations in *CDKL5* gene may lead to the production of a residual amount of normal mRNA. We can therefore speculate that the severity could depend on the proportion of functional CDKL5 produced by the mutated alleles.

In conclusion, our data confirm that *CDKL5*-associated encephalopathy is characterized by specific features that show an overlap with atypical RTT with early onset seizures. The key clinical features of this disorder highlighted in this study are early epilepsy with very frequent seizures, an interictal EEG pattern that is normal or shows background, severe hypotonia, poor eye contact, a three-step pattern epilepsy in most cases that includes infantile spasms and some RTT-like features, such as secondary deceleration of head growth, severe motor impairment, sleep disturbances, hand apraxia and hand stereotypies. All together, these features should lead the clinician to consider *CDKL5* molecular screening.

Supplementary material

Supplementary material is available at *Brain* online.

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