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Recurrent Mutations in the *CDKL5* Gene: Genotype-phenotype Relationships

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Mutations in the cyclin-dependent kinase-like 5 gene (*CDKL5*) have been described in epileptic encephalopathies in females with infantile spasms with features that overlap with Rett syndrome. With more than 80 reported patients, the phenotype of *CDKL5*-related encephalopathy is well-defined. The main features consist of seizures starting before 6 months of age, severe intellectual disability with absent speech and hand stereotypies and deceleration of head growth, which resembles Rett syndrome. However, some clinical discrepancies suggested the influence of genetics and/or environmental factors. No genotype–phenotype correlation has been defined and thus there is a need to examine individual mutations. In this study, we analyzed eight recurrent *CDKL5* mutations to test whether the clinical phenotype of patients with the same mutation is similar and whether patients with specific *CDKL5* mutations have a milder phenotype than those with other *CDKL5* mutations. Patients bearing missense mutations in the ATP binding site such as the p.Ala40Val mutation typically walked unaided, had normocephaly, better hand use ability, and less frequent refractory epilepsy when compared to girls with other *CDKL5* mutations. In contrast, patients with mutations in the kinase domain (such as p.Arg59X, p.Arg134X, p.Arg178Trp/Pro/Gln, or c.145 + 2T > C) and frameshift mutations in the C-terminal region (such as c.2635_2636delCT) had a more severe phenotype with infantile spasms, refractory epileptic encephalopathy, absolute microcephaly, and inability to walk. It is important for clinicians to have this information when such patients are diagnosed.

Key words: Rett syndrome; *CDKL5*; epileptic encephalopathy

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INTRODUCTION

Rett syndrome (RTT; OMIM#312750) is a devastating X-linked neurodevelopmental disorder characterized by a wide spectrum of clinical manifestations. Beside the classic RTT form, several RTT clinical variants have been described, including the early seizure variant (seizure onset before regression) [Neul et al., 2010]. The early seizure variant was initially described by Hanefeld [1985], in a

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girl with infantile spasms with hypsarrhythmia in her early development (see also [Rajaei et al., 2011]). Since the first description of X-chromosomal breakpoints disrupting the cyclin-dependent kinase-5 (*CDKL5*) gene in patients with severe early onset infantile spasms with hypsarrthythmia and profound global developmental arrest [Kalscheuer et al., 2003], point mutations in the *CDKL5* gene have been identified in patients with the early onset RTT variant or with seizures before 6 months of age [Tao et al., 2004; Weaving et al., 2004; Evans et al., 2005; Scala et al., 2005; Archer et al., 2006].

The phenotypes associated with *CDKL5* mutations range from a mild form with controlled epilepsy and ability to walk to a severe form with absolute microcephaly, virtually no motor development, and refractory epilepsy [RE; Bahi-Buisson et al., 2008b]. Because of the small number of patients in prior studies, genotype–phenotype correlation has been limited. It has been suggested that mutations of the N-terminal catalytic domain are associated with earlier onset and intractable infantile spasms followed by late onset multifocal myoclonic epilepsy, and that patients with stop codon mutations have a milder phenotype than those with missense or splicing mutations [Bahi-Buisson et al., 2008a; Russo et al., 2009].

During the last 3 years, we screened the *CDKL5* gene in more than 350 unrelated females with epileptic encephalopathy from pediatric neurology centers. Here, we report on recurrent *CDKL5* gene mutations identified in our cohort of patients, and examine whether the phenotype of patients with the same *CDKL5* mutation is similar and whether specific *CDKL5* mutations lead to a milder phenotype. Because the *CDKL5* gene is subject to X-chromosome inactivation (XCI), we also studied XCI to find if skewing could explain phenotypic variability in females heterozygous for the same mutated allele.

MATERIAL AND METHODS

Patients Ascertainment and Determination of Phenotypes

A group of 358 unrelated females with encephalopathy and early seizures were referred by various pediatric neurology centers to our diagnostic laboratory at Cochin Hospital in Paris for *CDKL5* analysis. The group included 200 individuals with severe encephalopathy (impairment of both motor functions and communication abilities) and RE without ISSX (infantile spasms syndrome, X linked) and no features of RTT, 80 patients with unexplained infantile spasms and 78 patients with encephalopathy with controlled epilepsy but with features that overlapped with atypical RTT (deceleration of head growth, stereotypies, autonomic features, and hand apraxia). All patients were non-familial. This study was prospectively reviewed and approved by our local research ethics committee.

Retrospective clinical history and comprehensive neurological examination data were either collected by the principal investigator or by physicians following the patients in this cohort. Phenotypic evaluation was performed with a specific attention to the early onset seizure variant criteria, as described [Bahi-Buisson et al., 2008b; Artuso et al., 2010]. For the purpose of this study, "severe intellectual disability" was defined as developmental skills that were sufficiently impaired to preclude formal cognitive testing.

CDKL5 Gene Mutation Screening by DHPLC

Genomic DNA was extracted using standard procedures from peripheral blood leukocytes. The coding region of *CDKL5* (RefSeq: NM_003159.2) was screened by denaturing high performance liquid chromatography (DHPLC). The PCR amplifications were performed as described [Bahi-Buisson et al., 2008b]. The melting temperatures for all primer pairs and the full list of DHPLC run temperatures have been described. The DHPLC was performed on a Wave nucleic acid fragment analysis system HSM (Transgenomic, Crewe, UK). The PCR products with abnormal DHPLC profiles were sequenced.

X Inactivation Studies

The analysis of XCI was performed as described by Allen et al. [1992]. X chromosome inactivation was considered significantly skewed if the ratio exceeded 75:25 [Weaving et al., 2003].

Statistical Analysis

Correlations of genotype and phenotype findings were evaluated using the Fisher's exact test. $P < 0.05$ was considered statistically significant.

RESULTS

Identification of Recurrent CDKL5 Mutations

In total, 26 different *CDKL5* mutations were identified among 358 girls presenting with encephalopathy and early seizures. In all cases, the mutations were absent in the parents, which indicated that these mutations were apparently de novo. Most mutations were unique but some recurrent mutations were identified. Eight recurrent mutations, previously described in the literature by our team or other groups, were identified in apparently unrelated patients (Fig. 1).

The most common mutation (five of the eight; 62%) was a transition at CpG dinucleotides (Table I).

The 12 patients identified with recurrent *CDKL5* mutations had a common clinical presentation consisting of severe epileptic encephalopathy starting within the first 6 months of life, with the development of severe intellectual disability with feature of RTT. All patients except one (Patient 2) had early onset seizures starting from 4 to 10 weeks of age (Table II). However, some clinical discrepancies emerged, suggesting that there may be a severe form and less severe presentation of *CDKL5*-related encephalopathy. These patients showed heterogeneous clinical presentations, including infantile spasms (6/12; 50%), refractory epileptic encephalopathy (7/12; 58%), absolute microcephaly (<3rd centile for OFC, 3/12; 25%) and ability to walk, whether requiring assistance or not (4/12; 33%).

Influence of the Mutation Type on the Severity of the Phenotype

To evaluate the influence of the specific *CDKL5* mutation on the severity of the phenotype, we compared the clinical presentation of patients bearing the same *CDKL5* mutation, focusing our attention

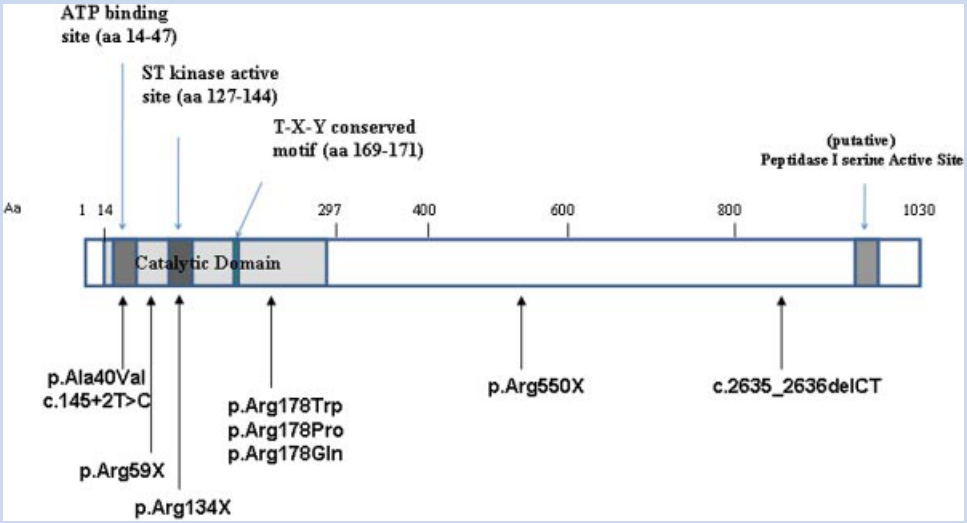

FIG. 1. Schematic illustration of CDKL5. Full-length human CDKL5 is 1030 amino acids long and contains within the catalytic domain the ATP-binding site, the serine-threonine (ST) kinase active site, and the conserved Thr–Xaa–Tyr (T–X–Y) motif. A putative signal peptidase I serine active site is indicated. The number at the top refers to the amino acid positions. The positions of recurrent point mutations identified in patients are illustrated.

TABLE I. Recurrent *CDKL5* Mutations Identified in our Cohort of Patients With Epileptic Encephalopathy

Name of the Mutation	Nucleotide substitution	Location	Affected Codon	No of patients	References
—	c.145 + 2T > C	Intron 4	G/gta	2	Bahi-Buisson et al. [2008b] Pintaudi et al. [2008] Russo et al. [2009]
p.Ala40Val	c.119C > T	Exon 4	GCG	5	Nemos et al. [2009] Mei et al. [2010] Melani et al. [2011] This study
p.Arg59X	c.175C > T	Exon 5	CGA	3	Archer et al. [2006] Ricciardi et al. [2009] Castren et al. [2011] This study
p.Arg178Trp	c.532C > T	Exon 8	CGG	3	Nemos et al. [2009] Artuso et al. [2010] This study
p.Arg178Pro	c.533G > C	Exon 8	CGG	1	Nemos et al. [2009]
p.Arg178Gln	c.533G > A	Exon 8	CGG	2	This study Liang et al. [2011]
p.Arg134X	c.400C > T	Exon 6	CGA	2	Rademacher et al. [2011] This study
p.Arg550X	c.1648C > T	Exon 12	CGG	3	Pintaudi et al. [2008] Russo et al. [2009] Rademacher et al. [2011] This study
p.Leu879GlufsX908	c.2635_2636delCT	Exon 18	CTG	3	Scala et al. [2005] Bahi-Buisson et al. [2008b] This study

TABLE II. Summary of the Clinical Features and the Identified Recurrent *CDKL5* Mutations in the Patients Reported in This Study

Patient	1	2	3	4	5	6	7	8	9	10	11	12
Mutation	p.Ala40Val	p.Ala40Val	p.Ala40Val[a]	p.Ala40Val[a]	p.Arg178Gln	p.Arg178Trp	p.Arg59X	p.Arg134X	c.145 + 2T > C[a]	p.Arg550X	c.2635_2636delCT	c.2635_2636delCT
Initial concern	ISSX	EE	AtyRTT	EE	EE	EE	AtyRTT	EE	AtyRTT	AtyRTT	EE	ISSX
Age at last evaluation	6yrs	12 yrs	3 yrs	6 yrs	6 months	11 yrs	21 yrs	6 yrs	5.5 yrs	4 yrs	2 yrs	5.2 yrs
Deceleration of head growth	+	+	−	−	−	+	+	+	+	−	−	−
Absolute microcephaly	−	−	−	−	−	+	−	+	+	−	−	−
Regression	−	−	+	−	+	+	−	+	−	−	−	−
Severe intellectual disability	+	+	+	+	+	+	+	+	+	+	+	+
Walk with aid or unaided	−	+	+	+	−	−	−	−	−	+	−	−
Limited hand skills	+	−	−	−	+	+	+	+	+	−	+	+
Hand stereotypies	+	−	−	+	−	+	+	+	+	+	+	−
Bruxism	+	−	−	+	−	+		+	−	+	+	−
Sleep disturbances	+	+	−	·	+		−	+	+	+	+	−
Seizure onset (weeks)	6	24	4	6	4	6	4	8	10	5	4	5
Infantile spasms	+	+	−	+	+	−	−	+	−	−	−	+
Late RE	+	−	−	−	+	+	−	+	+	−	+	+

EE, epileptic encephalopathy; yrs, years; AtyRTT, atypical RTT; +, presence.
[a]Mutations previously reported in Bahi-Buisson et al. [2008b] and revisited in this report.

on the four variable clinical criteria, previously described. The recurrent mutations identified in this study affected different domains of the CDKL5 protein, such as the ATP binding region, the serine–threonine protein kinase active site, the Thr–Xaa–Tyr sequence, the putative signal peptidase I serine active site, and a large COOH-terminal extension of almost 700 amino acids, poorly characterized, that probably harbors several functions [Bertani et al., 2006].

Recurrent Mutations Located in the ATP Binding Site

The ATP binding motif is a specific sequence of protein subunits that promotes the attachment of ATP to CDKL5. Modeling suggested that the Alanine at position 40 may interact with ATP. The recurrent missense mutation p.Ala40Val affects this conserved motif and likely abrogates ATP binding. As a result, this mutation may destroy the kinase activity [Hanks et al., 1988]. Here, we report on three novel unrelated patients bearing the p.Ala40Val mutation, increasing the number of patients to five [Rosas-Vargas et al., 2008; Bahi-Buisson et al., 2008b; Nemos et al., 2009; Melani et al., 2011]. Three patients were previously described (patients 16 and 18 in Bahi-Buisson et al. [2008b]; one patient in Mei et al. [2010]; Melani et al. [2011]). All patients except one (Patient 1 diagnosed with ISSX) were initially diagnosed with early onset epileptic encephalopathy. At last evaluation (median age 5 years, range 3–12 years), prominent clinical features in the four patients bearing the p.Ala40Val mutation were autistic features with poor eye fixation and pursuit. Half of these patients developed hand stereotypies. Head growth was normal range in all patients, but two had deceleration of head growth without absolute microcephaly. The mean age of seizure onset was 10 weeks (4–24 weeks). Only one of these patients developed refractory seizures. All had severe intellectual disability with absent language, but had relatively preserved gross motor skills with three patients (75%) able to walk unaided. The patient who was unable to walk (Patient 1) also had polymorphic refractory seizures. In the only other published case [Mei et al., 2010; Melani et al., 2011], the patient, aged of 1 year, had no head control, started seizures at day 38, and developed infantile spasms. However, because of her young age, no conclusion about the severity of her motor and epilepsy phenotype was possible. When we compared girls bearing the p.Ala40Val mutation to girls with other *CDKL5* mutations, those bearing the p.Ala40Val mutation had better hand use ability ($P = 0.03$), and tend to present a better ability to walk unaided ($P = 0.06$). We have found no evidence to suggest that the mild phenotype in girls with p.Ala40Val is related to non-random X inactivation patterns (Table III).

Recurrent Mutations Located in the Serine–Threonine Catalytic Domain

CDKL5 contains a conserved kinase catalytic domain. The Arg178-codon, lying within the kinase subdomain VIII (YVATRWYR), which is important for substrate recognition [Hanks and Hunter, 1995], appears to be a hot spot for *CDKL5* point mutations. Recurrent missense mutations have been identified at this codon (p.Arg178Pro, p.Arg178Trp, and p.Arg178Gln) [Artuso et al., 2010;

TABLE III. X Chromosome Inactivation (XCI) Pattern in *CDKL5* Mutation Patients

Patients (#)	Mutation	XCI (HpaII)
2623	p.Ala40Val	47:53
1807	p.Ala40Val	43:57
PA	p.Ala40Val	NI
BD	p.Ala40Val	NI
1251	p.Arg59X	51:49
863	c.145 + 2T > C	52:48
1461	p.Arg134X	75:25
2261	p.Arg178Trp	60:40
2658	p.Arg178Gln	53:47
1904	p.Arg550X	25:75
2560	c.2635_2636delCT	66:34
1015	c.2635_2636delCT	51:49

A skewing pattern was defined as greater than 75% of one X active allele. NI, non informative due to homozygosity for the CAG repeats of the androgen receptor gene.

Nemos et al., 2009; Liang et al., 2011], and here we describe two novel missense mutations affecting this amino acid (p.Arg178Trp and p.Arg178Gln). It is likely that exchange of a positively charged arginine (Arg) for an uncharged amino acid (Trp, Pro, and Gln) would likely influence substrate-binding specificity [Canagarajah et al., 1997].

A p.Arg178Gln mutation was identified in a 6-month-old girl. She was referred for epileptic encephalopathy starting at 4 weeks of age. She developed progressive infantile spasms that became refractory to antiepileptic drugs. Her motor development was severely delayed as was her eye contact. Her head growth was normal at 6 months and hand stereotypies were absent. This mutation was also identified in a boy aged 1 year and 9 months [Liang et al., 2011]. He also had epileptic spasms, and developed RE without absolute microcephaly. He was unable to sit or to walk.

We identified another missense mutation affecting the same codon (p.Arg178Trp) in an 11-year-old girl. Seizures started at 6 weeks with tonic seizures. She also developed progressive, RE but never had epileptic spasms. She also had severe motor impairment and was only able to hold her head at last evaluation (10 years old). She had absolute microcephaly and poor eye contact. She had bruxism but never developed hand stereotypies. Artuso et al. [2010] reported the same mutation in a patient with RE, deceleration of head growth without absolute microcephaly, poor eye contact, and midline stereotypies.

Finally, a third distinct mutation (p.Arg178Pro) was identified at the same codon [Nemos et al., 2009]. The girl bearing this mutation also developed epileptic spasms and RE. Altogether, these five patients demonstrated similar clinical features consisting of early onset seizures progressively evolving into RE, severe motor impairment with virtually absent motor development. We found no evidence that skewed X inactivation explained the severity of the phenotype (Table III).

Several recurrent truncating mutations have been also identified in the catalytic domain of CDKL5. Here, we describe three novel cases of mutations affecting this region, the mutation p.Arg59X, the mutation p.Arg134X, and the splice mutation c.145 + 2C > T.

The mutation p.Arg59X was initially described by Archer et al. [2006] in a 6 year-old girl but without detailed clinical data. Castren et al. [2011] identified another girl aged of 6 years bearing the same mutation, and here, we report a third patient aged of 21 years. The two previously reported patients were severely impaired. One was not able to sit, and the patient reported here was only able to sit with aid. Although the patient reported here and the patient described by Castren et al. [2011] had autistic features, hand stereotypies, and deceleration of head growth, their epilepsy presentation was different. The present patient started seizures at 4 weeks of age and never experienced seizures after this period, while the other patient had severe epilepsy with a seizure onset at 12 hr of life, evolving to infantile spasms and RE [Castren et al., 2011].

The p.Arg134X mutation was also recently described [Rademacher et al., 2011]. Combining the present patient with this previous report, both patients displayed severe encephalopathy at 6 years of age. The present patient was referred for early onset epileptic encephalopathy. She developed absolute microcephaly, and absent ambulation although she was able to sit. Seizures started at 8 weeks of age and progressively evolved into infantile spasms and RE.

Finally, the splice mutation c.145 + 2T > C was identified in two patients. The patient identified here was 5.5 years old and was referred for early onset epileptic encephalopathy. Seizures started at 10 weeks of age, but these were easily controlled with antiepileptic drugs. She developed deceleration of head growth but no absolute microcephaly. At last evaluation (5 years old), she was able to sit, had poor eye contact and stereotypies. However, this phenotype contrasts with the previous described case with seizures onset at 2.5 months evolving to infantile spasms and RE [Pintaudi et al., 2008; Russo et al., 2009].

Recurrent Mutations Located in the Large C-Terminal Domain of the Protein

Since the first description of the p.Arg550X mutation located between the two NLS domains [Pintaudi et al., 2008; Russo et al., 2009], two subsequent patients were described including a one in this report [Russo et al., 2009; Rademacher et al., 2011]. The patient reported here with the p.Arg550X mutation is now aged 4 years. Her seizures started at 5 weeks of age. These seizures transiently stopped at 6 months but recurred at 9 months. She became seizure-free from the age of 3.5 years. At last evaluation (4 years old), she was able to walk independently and was able to manipulate. Her head growth was normal and she had poor eye contact. She also developed bruxism, hand stereotypies, and episodes of hyperventilation. Both patients had similar epilepsy outcome, with seizure control and relatively preserved motor development since one is able to walk unaided at 4 years of age. However, head growth was normal in one case, while the other was severely impaired [Pintaudi et al., 2008].

The final recurrent mutation identified in this report was c.2635_2636delCT. This mutation was initially described by Scala et al. [2005] (see also [Buoni et al., 2006; Artuso et al., 2010], and was found in another patient in our previous study [Bahi-Buisson et al., 2008b]. The patient with this mutation reported here, aged of 5 years and 2 months, was referred for epileptic encephalopathy.

Seizures started at 5 weeks of age and also evolved into RE, but with infantile spasms. At last evaluation (5 years old), head circumference was normal, but she was unable to stand or sit unaided. She demonstrated no hand stereotypies or bruxism. Altogether, the patients bearing the c.2635_2636delCT mutation had features consisting of RE and severe motor disability; none were able to walk.

DISCUSSION

Since the first description of point mutations in *CDKL5*, more than 80 different point sequence variations have been described resulting in missense, nonsense, splice, and frameshift mutations [Tao et al., 2004; Weaving et al., 2004; Evans et al., 2005; Mari et al., 2005; Scala et al., 2005; Archer et al., 2006; Bahi-Buisson et al., 2008a,b; Pintaudi et al., 2008; Nemos et al., 2009; Sprovieri et al., 2009; Russo et al., 2009; Rademacher et al., 2011; Rajaei et al., 2011; Castren et al., 2011; Intusoma et al., 2011; Liang et al., 2011]. These mutations are distributed throughout the *CDKL5* coding sequence with missense almost exclusively found in the kinase domain. This mutational heterogeneity may in part explain the phenotypic heterogeneity. To evaluate this question, we examined the phenotype of patients bearing a number of recurrent mutations in several functional domains of CDKL5. We identified eight recurrent *CDKL5* mutations, 62% being located in CpG dinucleotides and corresponding to a C to T transition.

We found that patients with mutations in the ATP binding domain (e.g., p.Ala40Val) tended to have a milder phenotype. They had significantly better hand use ability and tended to preserve the ability to walk unaided. Similarly, patients with nonsense mutations in the C-terminal region of CDKL5 (e.g., p.Arg550X) had a milder phenotype. On the contrary, patients with mutations in the kinase domain and frameshift mutations located at the end of the C-terminal region had a more severe motor impairment, RE, stereotypies, and absolute microcephaly. These data support previous reports that mutations of the N-terminal catalytic domain were associated with a more severe phenotype, and that patients with nonsense mutations had a milder phenotype than those with missense or splicing mutations [Bahi-Buisson et al., 2008a; Russo et al., 2009].

Previous attempts to pool mutations according to their type or their locations within the *CDKL5* gene did not explain the observed phenotypic variability. The behavior of each missense, nonsense, and frameshift mutation might be different. First, proteins with some catalytic domain missense mutations are highly represented in the cytoplasm while those with nonsense mutations in the C-terminus are constitutively localized in the nucleus [Bertani et al., 2006; Rosas-Vargas et al., 2008; Rusconi et al., 2008]. Second, some missense mutations show a partial or complete loss of catalytic function, while other truncations lead to an increase in phosphorylation capacity [Bertani et al., 2006]. Third, each mutation may have a different effect on the *CDKL5* mRNA and protein levels. Some nonsense mutations may reduce mRNA level [Russo et al., 2009], while others may increase the protein stability and catalytic stability [Williamson et al., 2012]. The clinical phenotype associated with each *CDKL5* mutation may be the result of the nuclear or cytosolic accumulation of CDKL5, of its ability to bind substrate(s) and of the residual catalytic activity of CDKL5.

Although no mRNA analysis was carried out, it is likely that early truncating mutations lead to a non-functional truncated kinase without catalytic domain explaining the severe clinical phenotype. Because the splice mutation c.145 + 2T > C induces exon 4 skipping, this predicts, p.Glu34LysfsX60 [Pintaudi et al., 2008; Bahi-Buisson et al., 2008b], which is associated with a severe clinical phenotype in these patients similar to those with early truncating mutations.

Interestingly, the c.2635_2636delCT mutation was associated with a severe presentation. This mutation may lead to protein truncation in position 908, after a short stretch of 29 aberrant amino acids, without affecting the catalytic domain or the NES domain. The phenotype contrasts with the milder phenotype in two patients bearing a premature stop codon occurring near the end of COOH-terminus reported in the literature [Psoni et al., 2010; Intusoma et al., 2011]. In one report, paternal DNA was not available for screening, and in the second report, the mutation was also found in normal intellect individuals. It remains to be established whether these late truncating mutations are truly pathogenic. Recently, a novel CDKL5 107 kDa isoform with an alternative C-terminus that terminates in intron 18 was identified in all analyzed tissues including the brain [Williamson et al., 2012]. Our results support the idea that mutations affecting this 107 kDa isoform are likely pathogenic and that mutations only affecting the large 115 kDa protein are likely not involved in neurological symptoms.

Although we observed a relative homogeneity of the clinical phenotype in patients bearing the same mutation, some clinical discrepancies emerged. Since RTT is an X-linked trait and the *CDKL5* locus is subject to X inactivation, variable X inactivation may lead to different phenotypes among patients who have the same mutation. In accordance with literature data, we found that all *CDKL5* mutation patients described in this report demonstrated a random pattern of XCI in blood leukocytes. Other studies also showed that the clinical heterogeneity in unrelated patients with *CDKL5* mutation may not be attributable to XCI [Nemos et al., 2009], although we cannot rule out preferential inactivation of the normal allele in brain tissues in patients with a severe phenotype, and that the leukocytes XCI pattern in may not reflect the brain XCI pattern.

To conclude, we observed in patients bearing a *CDKL5* mutation, a common clinical presentation consisting of severe epileptic encephalopathy. However, patients bearing missense mutations in the ATP binding site (e.g., p.Ala40Val) had better hand use ability and an ability to walk unaided. In contrast, patients bearing mutations located in the kinase domain and frameshift mutations located in the C-terminal region of CDKL5 had a more severe phenotype.

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