

Cyclin-Dependent Kinase-Like 5 (*CDKL5*) Mutation Screening in Rett Syndrome and Related Disorders

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Rett syndrome (RTT) is a severe neurodevelopmental disorder affecting females almost exclusively and is characterized by a wide spectrum of clinical manifestations. Mutations in the X-linked methyl-CpG-binding protein 2 (*MECP2*) gene have been found in up to 95% of classical RTT cases and a lesser proportion of atypical cases. Recently, mutations in another X-linked gene, *CDKL5* (cyclin-dependent kinase-like 5) have been found to cause atypical RTT, in particular the early onset seizure (Hanefeld variant) and one female with autism. In this study we screened several cohorts of children for *CDKL5* mutations, totaling 316 patients, including individuals with a clinical diagnosis of RTT but who were negative for *MECP2* mutations ($n = 102$), males with X-linked mental retardation ($n = 9$), patients with West syndrome ($n = 52$), patients with autism ($n = 59$), patients with epileptic encephalopathy ($n = 33$), patients with Aicardi syndrome ($n = 7$) and other patients with intellectual disability with or without seizures ($n = 54$). In all, seven polymorphic variations and four *de novo* mutations (c.586C>T [p.S196L]; c.58G>C [p.G20R]; c.2504delC [p.P835fs]; deletion of exons 1 - 3) were identified, and in all instances of the latter the clinical phenotype was that of an epileptic encephalopathy. These results suggest that pathogenic *CDKL5* mutations are unlikely to be identified in the absence of severe early-onset seizures and highlight the importance of screening for large intragenic and whole gene deletions.

Keywords: *CDKL5*, Rett syndrome, infantile spasms, epileptic encephalopathy, intellectual disability, mutation

Rett syndrome (RTT; OMIM#312750) is an X-linked dominant neurodevelopmental disorder that manifests predominantly in females with profound intellectual impairment (Hagberg et al., 1983), and has an estimated incidence of 1 in 8,500 by the age of 15 years (Laurvick et al., 2006). This disorder is characterized by a wide spectrum of phenotypic severity (Hagberg et al., 2002).

In 1999, mutations in the X-linked gene methyl-CpG-binding protein 2 (*MECP2*) gene were identified (Amir et al., 1999), and it is now recognized that *MECP2* mutations may be found in 90 to 95% of individuals with classical RTT (Williamson & Christodoulou, 2006). Recently, mutations in another X-linked gene, cyclin-dependent kinase-like 5 (*CDKL5*) have been found to cause a RTT-like phenotype with early onset seizures and profound intellectual impairment (Bahi-Buisson, et al., 2008; Bahi-Buisson, et al., 2008; Evans et al., 2005; Scala et al., 2005; Tao et al., 2004; Weaving et al., 2004).

There are features of various disorders associated with intellectual disability that overlap with RTT, suggesting that these could be candidates for *CDKL5* mutation screening. Mutations in the *CDKL5* gene have been found particularly in the Hanefeld variant of RTT, with characteristic early-onset seizures, usually of the infantile spasm type (Scala et al., 2005).

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A male with cognitive impairment and seizures was found to have a large deletion that disrupted the *CDKL5* gene (Huopaniemi et al., 2000). Moreover, Tao et al. suggested that *CDKL5* may be associated with clinical features that overlap with those of other neurodevelopmental disorders such as autism and Angelman syndrome (Tao et al., 2004), and one patient in our original cohort with a pathogenic *CDKL5* mutation had autism but no seizures (Weaving et al., 2004). These observations, coupled with the previous observation that disruption of the *CDKL5* gene can be associated with X-linked infantile spasms (ISSX) (Kalscheuer et al., 2003), and the more recent finding of a *CDKL5* encephalopathy in which females have severe epilepsy and poor neurocognitive development without a RTT phenotype (Archer, et al., 2006), prompted the speculation that *CDKL5* mutations may underlie other clinical phenotypes.

In this study we explored whether *CDKL5* mutations may be associated not only with RTT but with other clinical disorders presenting with cognitive impairment, social dysfunction and/or seizures.

Materials and Methods

Patients

A total of 316 patients were selected for *CDKL5* screening. The cohort consisted of *MECP2* mutation-negative RTT patients, mainly with atypical variants, and patients with other clinical features that overlap with RTT.

Clinical Cohorts

RTT. The RTT group formed 36% of the patients recruited for this study. All samples were previously negative for mutations of *MECP2* gene. Mutation testing included screening all four exons of *MECP2* and MLPA studies for gene deletions or duplications. Of the 102 RTT patients, 10 had the classical phenotype and 92 had atypical RTT. Seizures occurred in 41 out of 67 patients for whom this information was available.

West syndrome. This heterogeneous group of patients consisted of 52 patients of whom 47 were males and 5 were females. The majority were sporadic cases. All patients were previously screened for mutations of the *ARX* gene, with no mutations identified.

Autism. The autism spectrum disorder group (59 subjects) included 4 females and 55 males. The males were recruited from the AGRE (Autism Genetic Resource Exchange) set and included only families with greater than one affected male, suggestive of X-linked inheritance. The vast majority of the affected males, within each family, shared a common haplotype across the Xp22 region.

Epileptic encephalopathy. A cohort of 30 females and 3 males with epileptic encephalopathies was studied. Inclusion criteria included refractory epilepsy with seizure onset under 6 months and cognitive impairment. The diagnosis of RTT had been entertained in

two cases; both were negative for *MECP2* mutations. 25 were negative for mutations of the gene encoding the alpha 1 subunit of the sodium channel, *SCN1A*, which is frequently mutated in Dravet syndrome.

Non-syndromic X-Linked Mental Retardation (XLMR). The nonsyndromic XLMR cohort numbered 9, and consisted of 1 female and 8 males and all had seizures.

Other related disorders. Seven female subjects had a clinical diagnosis of Aicardi syndrome. They all had the ocular anomalies typical of Aicardi syndrome, as well as agenesis or dysgenesis of the corpus callosum and seizures. The 54 remaining patients (8 males and 46 females) were incompletely classified but had clinical features of interest according to the selection criteria for this study, including intellectual impairment and 9 subjects had seizures.

Methods

DNA was extracted using the 'salting out' method (Miller et al., 1988). The 20 coding exons of *CDKL5* (exons 2–21) were amplified by PCR amplification of genomic DNA. Each reaction (volume 25 μ l) contained 25 ng of genomic DNA, 0.5 μ M of each primer, 0.2 mM of dNTPs, 0.75 U HotMaster Taq DNA Polymerase (Eppendorf, North Ryde, NSW, Australia), 1x HotMaster buffer with a final concentration of 2.5 mM MgCl₂. The thermocycling conditions were as follows: 94°C for 2 minutes (1 cycle); 94°C for 20 sec, 65°C for 10 sec, 70°C for 20 sec (2 cycles); 94°C for 20 sec, (63°C to 55°C, [decreased by 2°C per cycle] for 10s per cycle), 70°C for 20 sec (35 cycles); 72°C for 4 min. Investigation of *CDKL5* transcripts identified an alternative isoform (Williamson S, personal communication), resulting in the need to redesign the exon 18 primers. Primer sequences used are available on request.

The 20 coding exons of *CDKL5* were screened by denaturing high performance liquid chromatography (dHPLC) and direct sequencing. The cohort of patients with epileptic encephalopathies was screened for mutations as previously described (Scala et al., 2005). Additional screening for identified mutations was performed by either restriction digests or custom TaqMan SNP Genotyping Assays (Applied Biosystems, Carlsbad, CA, USA). The c.586C>T (p.S196L) variation leads to the loss of an *Ahd* I restriction site, the size of the uncut product is 280 bp, whereas in the wildtype PCR product two bands of 60 and 220 bp are produced. Control alleles and parental samples were therefore screened for the p.S196L missense mutation using a *Ahd* I restriction digest. Screening for the c.554+11G>A sequence variation was carried out using a *BsmF* I (New England Biolabs, Ipswich, MA, USA) restriction digest. A custom TaqMan SNP Genotyping Assay was used to screen for the c.978-23T>C variation.

Long-range PCR and cloning were carried out to determine whether the p.S196L mutation was located

on the same allele as c.554+11G>A. Both variations were amplified within one amplicon around 4 kb in length using Expand Long Template PCR System (Roche Applied Science, Castle Hill, NSW, Australia) according to manufacturer's instructions. PCR cloning was carried out using CopyControl cDNA, Gene & PCR Cloning kit (Epicentre, Madison, WI, USA) with minor changes to manufacturer's instructions.

Large deletions of the *CDKL5* gene were screened for using real-time quantitative PCR (QPCR) using either the same primers as used in DHPLC or new primers designed using Primer3 software (Rozen & Skaletsky, 2000). Glyceraldehyde-3-phosphate dehydrogenase was used as a genomic reference. Primer sequences are available upon request. Each reaction of 10 µl contained 40 ng of genomic DNA, 0.5 µM of each primer, 0.2 mM of dNTPs, 0.5 U *Taq* polymerase (New England Biolabs, Ipswich, MA, USA), 1x ThermoPol buffer with a final MgCl₂ concentration of 2.0 mM and 1x SYBR green (Invitrogen, Carlsbad, CA, USA). Thermocycling was carried out in a RotorGene 3000A thermocycler (Corbett Life Science, now QIAGEN, Doncaster, Victoria, Australia) with the following cycling conditions: an initiation denaturation of 94°C for 4 min, followed by 40 cycles of 94°C for 20 sec, 56°C for 20 sec and 70°C for 20 sec. Analyses of results were carried out using the accompanying software.

Results

Sequencing

The entire coding region of the *CDKL5* gene (exons 2 to 21) was screened by direct sequencing in our cohort of patients. Seven polymorphic variations and four probably or potentially pathogenic mutations were identified (Table 1).

c.58G>C (p.G20R) Missense Mutation

This 7-year-old girl had an epileptic encephalopathy. She had intractable seizures, profound early onset developmental regression, feeding difficulties requiring gastrostomy insertion, generalized hypotonia, precocious puberty and cortical visual impairment.

This girl was the younger of two children to unrelated Australian parents. The pregnancy and perinatal history were normal. Her development was normal until 4 months when she regressed. Her head control deteriorated, she smiled and vocalized less than previously, and she did not fix and follow as well. She then made some slow developmental gains but regressed again at 3.5 years. She sat at four years but has never walked. She had cortical visual impairment, however she would look at faces and follow people around a room. Rather than look directly at an object or person she tended to observe from the corners of her eyes.

Seizures started at two months of age, shortly after her first immunization. The seizures comprised epileptic spasms, partial tonic seizures with asymmetric posturing and myoclonic seizures. A vagal nerve stim-

ulator was inserted at 4 years, which improved seizure frequency and severity. At 7 years, her seizures remained refractory on a combination of clobazam and topiramate.

At 6 months, video-EEG monitoring showed subtle spasms progressing to a tonic seizure with extension of the right upper and lower limbs and flexion on the left side, evolving to a series of spasms. The ictal rhythm comprised non-lateralizing low amplitude fast activity in the frontal region spreading to the central region. The spasms were associated with bilateral frontotemporal discharges. At 5.5 years, her EEG background showed intermittent slowing with frequent trains of left frontotemporal discharges with independent discharges in the frontal and temporal regions bilaterally. MRI brain was normal.

At 5 years, she had precocious puberty with the onset of breast and pubic hair development and an early growth spurt.

On examination at 7 years, her head circumference was 49 cm (< 2nd percentile). She had subtle dysmorphic features with a full lower lip, straight eyebrows and deep-set eyes. She had a café au lait macule over her right ankle. She had small feet, her left foot length was 15.5 cm (< 3rd percentile) and her peripheries were warm and well perfused. She demonstrated midline hand clasping and hand mouthing movements. She showed limited visual fixation. Her breathing pattern was normal and her back was straight. Cardiovascular and respiratory examinations were also normal. Her general muscle mass was normal. She had generalized hypotonia, with intermittent tremulousness and dystonic posturing of her feet. She had a gastrostomy button in situ. In ankle/foot/knee orthoses, she was able to stand with assistance.

She was found to be heterozygous for the missense mutation, c.58G>C in exon 2, which changes the amino acid glycine (nonpolar neutral side chain) to arginine (polar positively-charged side chain) at position 20 of the protein (Figure 1a). The mutation was not present in either parent or 200 control alleles. Figure 1b shows the alignment of the human wild-type and the p.G20R mutant *CDKL5* sequence against eight *CDKL5* orthologs. The region is highly conserved, and is part of the ATP binding site of the putative kinase domain, common to seven *CDKL5* orthologs, as well as other members of the mitogen-activated protein (MAP) kinase and cyclin-dependent kinase (CDK) families (Montini et al., 1998). Disruption of the sequence at the kinase domain may potentially reduce the efficiency of the kinase activity of *CDKL5*, and this mutation is highly likely to be the pathogenic cause of the epileptic encephalopathy in this patient.

c.586C>T (p.S196L) Missense Mutation

The missense c.586C>T variation was identified in a female, the first child of nonconsanguineous parents. The patient was born at full term, and the perinatal period was uneventful. Normal development occurred

Table 1
Screening Results

Type of variant	Nucleotide	Amino acid change	No. of change	Diagnosis	Detected in Patients	Comments Parent
Missense	c.58G>C	p.G20R	1	EE (female)	Not found in either parent. Not present in 200 control alleles	Probably pathogenic.
Missense	c.[554+11G>A; 586C>T]	p.S196L	1	EE (female)	Not found in either parent. Not present in 300 control alleles. Intronic variation inherited from the father.	Missense variation is probably pathogenic. Intronic variation is believed to be nonpathogenic.
Frameshift	c.2504delC	p.P835fs	1	EE (female)	Not found in either parent	Probably pathogenic.
Deletion	Exons 1 – 3 (and 5' UTR)	No CDKL5 protein predicted	1	EE (female)	Not found in either parent	Pathogenic.
Missense	c.2372A>C	p.Q791P	19	WS (3), RTT (15). EE (1;female)	ND Seen in the father	Known c.SNP (Kalscheuer et al., 2003; Tao et al., 2004)
Intronic	c.2376+118T>A (IVS16+118T>A)	NP	21	WS (3), RTT (18).	ND	Known c.SNP (Evans et al., 2005)
Intronic	c.2377-31T>C (IVS16-31T>C)	NP	1	EE (1; female)	Heterozygous in unaffected mother. Not present in 200 control alleles.	(not reported.) Probable polymorphism
Intronic	c.978-23T>C (IVS11-23T>C)	NP	1	Male, seizures, MR	Heterozygous in unaffected mother. Not present in 306 control alleles.	Probable polymorphism
Intronic	c.463+22T>C (IVS7+22T>C)	NP	1	RTT, seizures	Heterozygous in unaffected mother. Not present in 306 control alleles.	Probable polymorphism (Evans et al., 2005)
Silent polymorphism	c.[3003C>T; 3084G>A]	p.[H1001H; T1028T]	3	Atypical RTT (1;female), Angelman-like (1; male), WS (1; male)	ND	Probable polymorphism (Huppke et al., 2005; Tao et al., 2004)

Note: MR – Mental retardation; RTT – Rett syndrome; WS – West syndrome; EE – epileptic encephalopathy; ND – not determined; NP – none predicted.

until 5 months of age when she could sit with support, reach for objects, had social smiling, recognition of her mother, good eye contact and nonspecific vocalization. Regression was noticed from the age of 5 months, when she no longer reached for objects, lost eye contact and had no useful hand activity. She developed hand-washing stereotypies and never produced any meaningful sounds. It was at this time that she developed infantile spasms, having 10–15 flexor spasms per day, which increased in frequency when she was drowsy. She was treated with ACTH, and subsequently with sodium valproate and clobazam without adequate control of seizures. When last

reviewed at the age of 3 years, she continued to have frequent seizures, had developed hypothyroidism and was on lamotrigine. Her breathing pattern was normal. Her head circumference was 47 cm (20% percentile), weight was 14 kg (50% percentile), and height was 94 cm (40% percentile). She also had small feet but had normal muscle tone. Her EEG showed hypsarrhythmia.

She was found to be heterozygous for the missense mutation c.586C>T in exon 9, which changes the amino acid serine (polar neutral side chain) to leucine (nonpolar neutral side chain) at position 196 of the protein (Figure 1a). Figure 1c shows a segment of the

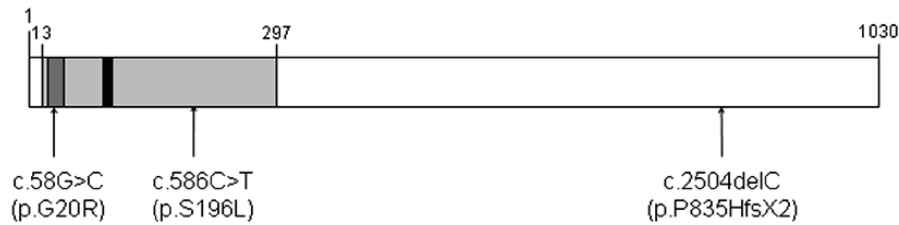


Figure 1a

Patient p.G20R	K F E I L G V V R E G A Y G V V
Homo sapiens NP_003150	K F E I L G V V G E G A Y G V V
Mus musculus NP_001019795	K F E I L G V V G E G A Y G V V
Danio rerio XP_687767	K F E V L G I V G E G A Y G V V
Monodelphis domestica XP_001380717	K F E I L G V V G E G A Y G V V
Tetraodon nigroviridis CAF99817	K F E V L G I V G E G A Y G V V
Gallus gallus XP_425571	K F E I L G V V G E G A Y G V V
Canis familiaris XP_548881	K F E I L G V V G E G A Y G V V
Macaca mulatta XR_009820	K F E I L G V V G E G A Y G V V

Figure 1b

Patient p.S196L	Y G K S V D M W L V G C I L G E
Homo sapiens NP_003150	Y G K S V D M W S V G C I L G E
Mus musculus NP_001019795	Y G K S V D M W S V G C I L G E
Monodelphis domestica XP_001380717	Y G K S V D M W S V G C I L G E
Danio rerio XP_687767	Y G K A V D M W S V G C I L G E
Tetraodon nigroviridis CAF99817	Y G K A V D M W S V G C I L G E
Takifugu rubripes AAD28798	Y G K A V D M W S V G C I L G E
Gallus gallus XP_425571	Y G K A V D M W S V G C I L G E
Canis familiaris XP_548881	Y G K S V D M W S V G C I L G E
Macaca mulatta XR_009820	Y G K S V D M W S V G C I L G E

Figure 1c

Figure 1

Positions of pathogenic CDKL5 mutations and Protein sequence alignments of CDKL5 orthologs.

1a: The catalytic domain of CDKL5 (light grey box) contains an ATP binding site (dark grey) and the serine-threonine kinase active site (black box). The positions of the two missense mutations (p.G20R and p.S196L) and the frameshift mutation (p.P835HfsX2) are indicated by arrows.

1b: Conservation of amino acid p.G20E across different CDKL5 orthologs. The glycine residue is invariant across all nine species and resides in a region that is well-conserved.

1c: Conservation of amino acid p.S196L protein sequence alignment of CDKL5 orthologs. The mutated serine at position 196 is invariant across all species for which protein sequence, within a highly conserved region of the protein.

Sequences were aligned using Geneious (Biomatters, Auckland, New Zealand), from the following GenBank sequences: *Homo sapiens* (NP_003150.1), *Mus musculus* (NP_001019795.1), *Monodelphis domestica* (XP_001380717.1), *Danio rerio* (XP_687767.3), *Tetraodon nigroviridis* (CAF99817.1), *Takifugu rubripes* (AAD28798.1), *Gallus gallus* (XP_425571.2), *Canis familiaris* (XP_548881.2) and peptide sequence predicted from *Macaca mulatta* mRNA sequence XR_009820.1.

protein kinase domain, demonstrating that the altered amino acid is highly conserved within this domain. The mutation was not present in either parent or 300 control alleles. It is likely that this mutation affects the protein kinase activity of *CDKL5*.

We also identified the c.554+11G>A (IVS8+11G>A) variation in the proband. We consider this previously unreported variation as non-pathogenic as it was also

identified in the unaffected father. Moreover, the Berkeley Drosophila Genome Project Splice Site Predictor (http://www.fruitfly.org/seq_tools/splice.html) predicted that this variation would not affect exon splicing. Long-range PCR and cloning using DNA from the proband was performed, the aim of which was to capture both this polymorphism and the missense mutation identified in the proband in the same fragment.

Screening of 10 colonies revealed that the polymorphism and missense mutation were on the same allele, i.e. the missense mutation arose on the X chromosome inherited from her father.

c.2504delC Frameshift Mutation

This 3.5-year-old girl was the second child of unrelated parents. She was the product of a normal pregnancy and delivery at 37 weeks gestation. She had gastro-esophageal reflux in the first 8 weeks of life. Seizures began at 8 weeks and comprised 5–10 second tonic seizures with peri-oral clonic activity when she was unwell with fever and vomiting. Seizures continued daily until 11 months when she had a 2-month seizure-free period. Subsequently seizures recurred in clusters, lasting 7–15 minutes and occurred during sleep. They were unusual attacks that began with coughing and gagging, she would cry ‘mum’, then would roll from side to side and develop stiffening and extension of all limbs with facial grimacing. She would gasp with slow rhythmic movements of her limbs. There was retained awareness with some seizures such that she would look for her mother and in more severe attacks, her eyes would deviate to the right or fix straight ahead. At 3.5 years, her parents elected not to have her on anti-epileptic therapy.

Developmentally, her head control was good at 15 weeks but she had not yet rolled over when she regressed at 25 weeks while on multiple anti-epileptic agents. She became less interactive, stopped fixing and her head control deteriorated. She rolled at 12 months, spoke single words at 18 months and sat at 2 years. At 21 months, she developed hand to mouth stereotypic movements. At 3 years 2 months, she had a few words, could follow simple commands and had started to crawl. She had a long history of constipation. Her head circumference was 50 cm (50th centile) and her weight was 14.1 kg (50th centile). She had bilateral epicanthic folds and an alternating convergent strabismus. She had generalized hypotonia with normal reflexes and downgoing plantar responses.

An EEG at 21 months showed a normal background. Ictal recordings showed unusual seizures that began with truncal automatisms involving her hips and legs. These movements evolved to a tonic cruciate posture followed by superimposed spasms that continued for up to 20 minutes. MRI brain was normal. FDG PET scan showed mild bitemporal hypometabolism.

She was heterozygous for a frameshift mutation c.2504delC in exon 18, which changes the amino acid proline at position 835 to a histidine, followed immediately by a stop codon (p.P835HfsX2). The mutation was not present in either parent, and we predict its effects to be pathogenic. The ensuing truncated protein would be missing a large part of the C-terminal end of CDKL5 (Figure 1a), which is important for the correct localization of CDKL5 and therefore is likely to impact on function (Rusconi et al., 2008).

Large Deletion, Including Exons 1–3 of CDKL5

This 7.5-year-old girl also had an epileptic encephalopathy. She was the third child to nonconsanguineous Italian parents. She was born after an uneventful pregnancy and birth. Her development was always delayed with no clear history of developmental regression. From 2 months of age she had infantile spasms. She continued to have intractable seizures despite anti-epileptic medications, corticosteroids and the ketogenic diet.

At approximately 6 months she developed midline stereotypic hand movements in the form of hand clasping, wringing and hand to mouth movements. She sat unsupported at 2 years of age but has never learnt to crawl or pull herself to stand. She never learnt to say any words. She now occasionally babbles, however, communicates mainly through body language and gestures. She can make choices by touching a desired object and can reach out and grasp an object. There has been no loss of purposeful hand function or communicative abilities. In fact, she has continued to make small developmental gains. In addition, she has a disturbed sleep pattern, suffers with constipation, has difficulty chewing and grinds her teeth.

When reviewed at 7.5 years, she had frequent seizures, mainly clusters of spasms. Her weight was 21.7 kg (< 30th percentile) and head circumference was 51.5 cm (50th percentile). She had some facial dysmorphism with full lips, eversion of the lower lip, a prominent columella and horizontal palpebral fissures. She demonstrated midline hand clasping, wringing and finger twisting movements. Her peripheries were warm and well perfused, her back was straight and her breathing pattern was normal. She had generalized hypermobility particularly at the hips, knees, elbows and ankle and pes planus. When looking at people or objects she tended to look from the corners of her eyes.

Sequencing of the *CDKL5* coding sequence failed to reveal any changes. Quantitative PCR of genomic DNA showed that exons 1–3 were deleted. Using the ‘Agilent aCGH’ 44K array the deletion was found to extend into the 5’ UTR region of the *CDKL5* gene. The ISCN description is arr cgh Xp22.13p22.13 (A_14_P_104234>A_14_P125155) × 1. The deletion is located at 18.44 & 18.55 Mb respectively. It has a minimum size of 0.11Mb and maximum size of 0.19 Mb. The patient was also tested on the ‘Cytochip’ BAC array platform. The results obtained were consistent with the above findings. Neither parent was found to have this deletion.

Missense polymorphic variation (c.2372A>C; p.Q791P)

A coding single nucleotide polymorphism (c.SNP) was detected at position 2372 (c.2372A>C), within exon 16, which results in the amino acid change p.Q791P. This c.SNP was previously detected in 20% of a panel of 50 control chromosomes (Kalscheuer et al., 2003; Tao et al., 2004). This variation was identified in 19 individuals (15 RTT; all female and 3 West syndrome patients; all male and 1 epileptic encephalopathy,

female). Of these patients 12 individuals were known to have seizures.

Intronic Variation c.2376+118T>A

The c.2376+118T>A (IVS16+118T>A) variation was identified in 21 patients in this cohort, 18 RTT (all female) and 3 West syndrome patients (all male). Of these patients 13 individuals were identified as having seizures. This variation has been previously reported as a common SNP detected in intron 16 with a minor allele frequency of 33% (Evans et al., 2005).

Intronic Variation c.2377-31T>C

The c.2377-31T>C (IVS16-31T>C) variation was identified in 1 patient, with an epileptic encephalopathy associated with a small hypothalamic hamartoma and a balanced translocation of chromosomes 3 and 14. Her clinically normal mother was also found to be heterozygous for this intronic variation. This sequence variation was not found in dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>), the Database of Genomic Variants (<http://projects.tcag.ca/variation/>) or in the Japanese SNP database (<http://snp.ims.u-tokyo.ac.jp/>). Moreover, the Berkeley Drosophila Genome Project Splice Site Predictor (http://www.fruitfly.org/seq_tools/splice.html) predicted that this variation would not affect exon splicing. We conclude that this variation is unlikely to be pathogenic.

Intronic Variation c.978-23T>C

The c.978-23T>C (IVS11-23T>C) variant was identified in a male individual, who had neonatal seizures followed by severe epilepsy in infancy. He has severe mental retardation and phenotypically displayed features of both RTT and Angelman syndrome, including laughing spells, hand stereotypies, aphasia, mild microcephaly, mild spasticity, broad mouth, and a sleep disorder. Serial MRI scans of the brain have been normal. Investigation for inborn errors of metabolism was negative. In addition cerebrospinal fluid levels of neurotransmitters, folic acid metabolites, purines and pyrimidines were normal. The patient was negative for mutations in *UBE3A*, *MECP2* and *ARX*.

The BDG Splice Site Predictor program predicted that this variation would not affect exon splicing. This previously unreported variation was also identified in the unaffected mother but not in 306 normal control alleles. On balance, given the splice prediction analysis, we feel that this variation is most likely non-pathogenic.

Intronic Variation c.463+22T>C

The c.463+22T>C (IVS7+22T>C) variant was identified in a female RTT individual, who had well-controlled seizures. This variation was also identified in the unaffected mother of this patient but not in 306 normal control alleles. The BDG Splice Site Predictor program predicted that this variation does not affect the exon splicing. This sequence variation was first reported by Evans and colleagues in 2005 (Evans et al., 2005) who also reported that it would

not cause any changes to the splicing prediction. It is therefore likely that this variation is nonpathogenic.

Silent Polymorphisms: c.[3003C>T; 3084G>A]

Two silent polymorphisms (c.3003C>T and c.3084G>A), occurring 81 base pairs apart, were identified in three patients, one female and two males. We note that these two silent variations have been previously reported (Huppke et al., 2005; Tao et al., 2004), forming a rare conserved haplotype (along with the IVS4+17A>G variation) that was found in 2 of 267 controls from Caucasian individuals (Tao et al., 2004). The BDGP Splice Site Predictor predicted that neither of these variations would create the potential for abnormal splicing. Therefore it is concluded that this combination of sequence variations is probably non-pathogenic.

Discussion

In recent studies *MECP2* mutations have been found in up to 90–95% of patients with classical RTT (Williamson & Christodoulou, 2006) and in a lesser proportion (20–60%) of those with atypical forms of RTT (Bourdon, et al., 2001; Buyse et al., 2000; Cheadle et al., 2000; Hoffbuhr et al., 2002). Further investigations of the remaining group of clinically well defined RTT patients led to the discovery of mutations in a second gene, *CDKL5* (Evans et al., 2005; Scala et al., 2005; Tao et al., 2004; Weaving et al., 2004). Disruption of the *NTNG1* gene was found in one female with RTT (Borg et al., 2005), but mutations in *NTNG1* do not appear to be a significant cause of RTT (Archer, et al., 2006). In addition, mutations in *FOXP1* were more recently reported to cause the congenital variant of RTT (Ariani et al., 2008; Mencarelli et al., 2009), and classical RTT in one report (Philippe et al., 2009).

We screened a cohort of patients for mutations in the *CDKL5* gene. In our study we identified four novel mutations that we consider probably or potentially pathogenic (Table 1). In addition, we identified seven polymorphic variations, three of which were also seen in unaffected parents, and are therefore likely to be rare polymorphisms. Three SNPs identified in this study were previously reported (Evans et al., 2005; Kalscheuer et al., 2003; Tao et al., 2004), as was the complex allele with two silent variations in *cis* (Huppke et al., 2005; Tao et al., 2004). None of the intronic variants are likely to affect splicing according to the prediction software used.

Two females with an epileptic encephalopathy clinical picture were found to have missense mutations. One was found to be heterozygous for a c.58G>C mutation in exon 2 leading to a glycine to arginine amino acid change at position 20. The substitution is located in the ATP-binding site of the putative kinase domain of *CDKL5*. The inclusion of the larger sidechain of arginine in place of the single hydrogen in glycine is therefore likely to disrupt the binding ability

at that position. A heterozygous mutation in exon 9 (c.586C>T, p.S196L) was identified in the second. We screened both parents of the second case and 300 nonethnically matched control alleles and none were found to have this sequence variation. In addition, this sequence variation was not found in dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>), the Database of Genomic Variants (<http://projects.tcag.ca/variation/>) or in the Japanese SNP database (<http://snp.ims.u-tokyo.ac.jp/>). Serine at position 196 of the *CDKL5* protein is a highly conserved residue located within the kinase domain (Figure 1c), and so could potentially affect the protein kinase activity of *CDKL5*. In addition, the substitution of the highly polar serine for the highly nonpolar leucine may affect the structure of the protein. We therefore suggest that this mutation is likely to be pathogenic. Functional studies to assess phosphorylation capability of this missense mutation are warranted.

One patient with an epileptic encephalopathy phenotype was found to have a frameshift mutation (c.2504delC; p.P835fs). The resultant peptide is predicted to contain only the first two amino acids encoded by exon 18, with a shortened C-terminal. The C-terminus of *CDKL5* is postulated to be of importance to its subcellular localization. A derived truncation of *CDKL5* (Δ 831) and two nonsense mutations (p.R781X, p.L879X), all of which result in peptides of similar length, show a disruption in export from the nucleus in human HeLa cells, with these forms to be entirely nuclear. In contrast, both wild-type *CDKL5* and a longer truncated version of *CDKL5* (Δ 941), shows a more cytoplasmic distribution, suggesting that the peptide sequence (or part thereof) between 879 and 941 may play a role in the localization of *CDKL5* (Rusconi et al., 2008). The C-terminus is also thought to be important in the regulation of *CDKL5* kinase activity (Lin et al., 2005). The loss of the entire C-terminal domain of *CDKL5* (from amino acid 353 onwards) has elevated protein probably due to increased stability and enhanced autophosphorylation activity. It is uncertain whether the same effects would be seen with this particular mutation (c.2504delC, p.P835fs) as the peptide would still retain a section of the C-terminus.

One patient, a girl who also had an epileptic encephalopathy clinical picture, was found to have a heterozygous deletion of exons 1–3 and up to 190 kb of upstream sequence, without disrupting any other genes upstream of *CDKL5*. A similar disruption has recently been reported in a girl with severe ISSX (Cordova-Fletes et al., 2009), whereby the balanced translocation resulted in exons 1–3 of *CDKL5* being transposed to chromosome 2. Exons 4–21 of the disrupted *CDKL5* were shown to be transcribed, although any translated peptide would be partially devoid of the critical N-terminal kinase domain and likely to be nonfunctional. The breakpoint in exon 3 of *CDKL5* in the ISSX patient is limited to within 9 to

21 kb downstream of the intron 3 donor splicing site. The breakpoints of the deletion in our patient were not determined, and it would be of interest to examine whether there might be a potential breakpoint hotspot in that region of the chromosome.

Presently, the diagnosis of RTT remains a clinical one and is not made solely on the basis of identification of *MECP2* or *CDKL5* mutations. However, *MECP2* and *CDKL5* mutations can occur in association with non-RTT phenotypes. To date, over 40 mutations in *CDKL5* have been reported (reviewed in Nemos et al., 2009). Genotype-phenotype correlations have been limited because very few of the mutations have been identified in more than 1 patient. It remains unclear why *CDKL5* mutations are associated with seizures, as to date, apart from MeCP2, phosphorylation targets of *CDKL5* are yet to be identified. Bahi-Buisson and colleagues recently investigated how genotype might influence the epilepsy outcome in patients with *CDKL5* mutations (Bahi-Buisson et al., 2008; Bahi-Buisson et al., 2008). They suggested that the course of epilepsy is more severe in patients who carry mutations in the catalytic domain of *CDKL5* as opposed to patients with late truncating mutations. Further genotype-phenotype correlations may yield further insights into functionally significant domains within the protein.

Four genes have been associated with phenotypes overlapping with RTT, namely *ARX*, *NTNG1*, *CDKL5* and *FOXG1*. Mutations in the *ARX* gene cause a broad range of more than 8 phenotypes including infantile spasms, X-linked lissencephaly with ambiguous genitalia (XLAG) and X-linked mental retardation (Gecz et al., 2006). *NTNG1* has been reported to be a candidate gene for a RTT-like phenotype (Borg et al., 2005), and *CDKL5* mutations are a significant cause of spasms and early epileptic encephalopathy (Archer et al., 2006; Bahi-Buisson et al., 2008). An MLPA kit has been devised to screen these three genes to detect large duplications and deletions that would be missed by sequencing. *MECP2* deletions occur in up to 30% of females with classical RTT who have no detectable *MECP2* mutations by sequencing or dHPLC (Archer et al., 2006; Ariani et al., 2004; Bourdon et al., 2001; Erlandson et al., 2001; Erlandson et al., 2003; Hardwick et al., 2007; Laccone et al., 2004; Ravn et al., 2005; Schollen et al., 2003). MLPA screening of the *CDKL5* gene has been reported only twice to date (Mei et al., 2009; Russo et al., 2009). We attempted to use this kit, but despite repeated attempts using DNA that had undergone whole genome amplification using the GenomiPhi amplification kit (GE Healthcare) we were not able to generate reproducible results (data not shown). For this reason, we used qPCR and identified a large deletion in one patient, with exons 1–3 and upstream sequence (also identified by array CGH), which we estimate to be 110–190 kb in size. Interestingly, Nemos and colleagues have very recently reported a patient with a somewhat smaller (43 kb) deletion involving exons 1, 1a and 1b of *CDKL5* and

upstream sequence (Nemos et al., 2009). In addition, Erez and colleagues reported 3 females with deletions ranging between 74 and 157 kb identified initially by array CGH, also involving the first 3 – 4 exons of the *CDKL5* gene (Erez et al., 2009). These findings highlight the importance of not relying solely on MLPA results for the identification of large rearrangements of the *CDKL5* gene, and this has been our experience with the *MECP2* gene as well (Hardwick et al., 2007). It will only be by performing *CDKL5* MLPA analysis on a large cohort with confirmatory testing using other methods that the importance of MLPA screening of this gene will be elucidated.

The first *CDKL5* abnormalities identified were in patients with ISSX who had X: autosome translocations disrupting the *CDKL5* gene (Kalscheuer et al., 2003). Most RTT cases reported to date with *CDKL5* mutations have been associated with an early onset seizure variant, the so-called *Hanefeld variant* (Evans et al., 2005; Scala et al., 2005; Tao et al., 2004; Weaving et al., 2004). Additionally, it is now recognized that *CDKL5* mutations may cause a severe infantile onset seizure disorder with poor cognitive development (Archer et al., 2006; Bahi-Buisson et al., 2008). However, our studies suggest that *CDKL5* mutations are rarely found in ARX mutation-negative individuals with West syndrome or autistic patients without seizures. We had previously reported a *CDKL5* mutation in a female with autism, her identical sister with the early onset seizure variant of RTT, and her brother with epileptic encephalopathy. Despite the twin sisters having the same X-chromosome inactivation pattern in peripheral blood, the possibility remains that the different clinical phenotypes may be the result of skewed X-inactivation in the brain in one twin. In addition, our data and the findings of others (Bahi-Buisson et al., 2008; Elia et al., 2008), strongly suggest that *CDKL5* screening of *MECP2* mutation-negative RTT patients is more likely to yield pathogenic mutations if they have early onset seizures, particularly an epileptic encephalopathy, and this notion is supported by the recent publications of Mei and colleagues (Mei et al., 2009) and Nemos and colleagues (Nemos et al., 2009). It remains to be seen whether individuals with milder phenotypes (Psoni et al., 2009) will be consistently found to have *CDKL5* mutations.

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