

ORIGINAL ARTICLE

CDKL5/STK9 is mutated in Rett syndrome variant with infantile spasms

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Background: Rett syndrome is a severe neurodevelopmental disorder, almost exclusively affecting females and characterised by a wide spectrum of clinical manifestations. Both the classic form and preserved speech variant of Rett syndrome are due to mutations in the *MECP2* gene. Several other variants of Rett syndrome have been described. In 1985, Hanefeld described a variant with the early appearance of convulsions. In this variant, the normal perinatal period is soon followed by the appearance of seizures, usually infantile spasms. We have observed two patients with signs of Rett syndrome showing acquired microcephaly and stereotypic midline hand movements. The disease started with generalised convulsions and myoclonic fits at 1.5 months in the first patient and with spasms at 10 days in the other, suggesting a diagnosis of the Hanefeld variant. In these patients, *MECP2* point mutations and gross rearrangements were excluded by denaturing high performance liquid chromatography and real time quantitative PCR. The *ARX* and *CDKL5* genes have been associated with West syndrome (infantile spasms, hypsarrhythmia, and mental retardation).

Methods: Based on the clinical overlap between the Hanefeld variant and West syndrome, we analysed *ARX* and *CDKL5* in the two girls.

Results: We found frameshift deletions in *CDKL5* in both patients; one in exon 5 (c.163_166delGAAA) and the other in exon 18 (c.2635_2636delCT). *CDKL5* was then analysed in 19 classic Rett and 15 preserved speech variant patients, all *MECP2* negative, but no mutations were found.

Conclusion: Our results show that *CDKL5* is responsible for a rare variant of Rett syndrome characterised by early development of convulsions, usually of the spasm type.

Rett syndrome (RTT; MIM# 312750) is a neurodevelopmental disorder characterised by a wide spectrum of clinical manifestations. In the classic form, after a period of normal development, patients show growth retardation and regression of speech, along with purposeful hand movements and appearance of stereotyped hand movements. RTT variants have been described, including the preserved speech variant (PSV), characterised by the recovery of some degree of speech; the congenital variant (recognised from birth); the "early seizure variant" (seizure onset before regression); and the "forme fruste", with a milder, incomplete clinical course (regression between 1 and 3 years).^{1–6} These variants present some symptoms of RTT, but show considerable variation in type and age of onset, severity of impairment, and clinical course. Among these, the "early seizure variant" was initially described by Hanefeld in 1985, who reported a girl with infantile spasms with hypsarrhythmia in her early development.³

Approximately 80% of patients with classic RTT have a mutation in the methyl CpG binding protein 2 gene (*MECP2*; OMIM #300005).^{7–9} *MECP2* mutations have also been identified in about 50% of PSV cases and in a lower percentage of other variants.^{8,10–12} In the variant with early development of convulsion described by Hanefeld, *MECP2* mutations have not been published.³

The Hanefeld variant of RTT presents a phenotypic overlap with West syndrome, also called infantile spasm syndrome, X linked (ISSX). ISSX is characterised by the triad of infantile spasms, hypsarrhythmia, and severe to profound mental retardation. Some families with ISSX carry mutations in the aristaless related homeobox (*ARX*) gene, which maps to Xp21.3–p22.1.^{13,14} *ARX* mutations cause several forms of

epilepsy, including infantile spasms, myoclonic seizures, and peripheral dystonia, as well as syndromic and non-syndromic X linked mental retardation.^{13,14} Recently, a second gene has been found to be involved in ISSX, the cyclin dependent kinase-like 5 gene (*CDKL5/STK9*; NM_003159).¹⁵ These authors characterised two unrelated female patients with an apparently balanced translocation, 46,X,t(X;7)(p22.3;p15) in one case and 46,X,t(X;6)(p22.3;q14) in the other. The two patients presented a similar phenotype, comprised of severe early onset infantile spasms with hypsarrhythmia and profound global developmental arrest. In both patients, the X chromosomal breakpoints disrupted *CDKL5*. As there is phenotypic overlap between the Hanefeld variant and ISSX, we tested both *ARX* and *CDKL5* for mutations in the two RTT patients with early onset of convulsions. The analysis was subsequently extended to 19 classic RTT and 15 PSV cases.

METHODS

Patients

We investigated two patients, aged 9 and 8 years respectively, with early development of convulsions, who later developed many characteristics of RTT. They both fulfilled the criteria for the early seizure variant of RTT.^{4,5,16}

We then investigated 19 classic RTT and 15 PSV patients. The girls with classic RTT were diagnosed according to the international criteria.¹⁷ The PSV girls fulfilled the criteria of Hagberg and Skjedal for RTT variants.⁵ In particular, the PSV

Abbreviations: DHPLC, denaturing high performance liquid chromatography; ISSX, infantile spasm syndrome, X linked; PSV, preserved speech variant; RS, retinoschisis; RTT, Rett syndrome

cases show the same clinical features and stages of RTT in the first years of life, but they subsequently improve in fine motor ability and start to speak with an increasing number of words and phrases.

Molecular analysis

Blood samples were obtained after informed consent. DNA was extracted from peripheral blood using a QIAamp DNA blood kit (Qiagen). DNA samples were screened for mutations in *ARX* and *CDKL5* using transgenomic WAVE denaturing high performance liquid chromatography (DHPLC). The *CDKL5* coding portion was entirely analysed using the primers and conditions given in table 1. PCR products resulting in abnormal DHPLC profiles were sequenced on both strands by use of PCR primers with fluorescent dye terminators on an ABI Prism 310 genetic analyser (PE Applied Biosystems, Foster City, CA, USA). PCR products of exon 5 and exon 18 were separated on 6% polyacrylamide gel to define exactly the deleted bases. Normal and mutant alleles were cut from the gel and sequenced individually. X inactivation studies were performed using the assay of Pegoraro *et al.* Intensity of silver stained bands was measured using the Diversity Database program (Biorad) and the values were corrected for preferential allele amplification.¹⁸ RNA isolation from lymphoblasts and cDNA synthesis were performed according to standard protocols.¹⁹ We used primers designed to form cDNA products spanning exons 4–6 (4RTf: 5'-GAAACA CATGAAATTTGTGGCG-3'; 6RTr:5'-GTGAATAGCCTTGATTAG CTG-3') and 17–18 (17RTf: GAGAAGATCTCAGATCTGCAG; 18RTr: AGCTGGAGGGCTGGCCTG). RT-PCR products were separated by electrophoresis through a 6% polyacrylamide gel and silver stained.

RESULTS

We observed two patients with early development of convulsions who later showed characteristics of RTT. The phenotype of each case is described below.

Patient 1

This patient is a girl, now aged 9 years. Her pedigree is represented in fig 1A (left). The mother had a normal pregnancy and delivery. The child was affected with slight cyanosis after birth. Birth weight was 3600 g and head circumference was 34 cm (50th centile). Generalised convulsions appeared at 1.5 months of age and were barely controlled by various antiepileptic drugs. In the following months she was examined in hospital, and myoclonic fits resembling infantile spasms were noted, although her EEG was not typical of hypsarrhythmia. Her developmental milestones were delayed and she was able to sit unaided at 1 year and to walk unaided at 6.5 years. She was examined again in hospital at the age of 2 years. MRI of the head, evaluations for aminoacidaemia, karyotype, search for Fragile X syndrome, methylation pattern for Angelman syndrome, and *UBE3A* gene sequencing were all negative. The patient was initially examined in our unit at the age of 8.5 years. She was able to briefly hold an object in her hands, dropping it shortly afterwards. She brought her hands frequently to her mouth, or beat them together. She had stereotypic hand-washing activities, reported as occurring since the age of 1 year. Her facial expression varied and she was able to interact at a pre-verbal level. She was able to utter one word and had occasional bruxism and hyperventilation. Her head circumference was 48.5 (<3rd centile). Scoliosis, kyphosis, cold extremities, and constipation were not present. The EEG showed sharp waves in the central and occipital regions.

Table 1 Primer sequences and conditions for PCR reaction and DHPLC analysis of *CDKL5* amplicons

Exon	Forward primer	Reverse primer	Product length (bp)	PCR annealing temp (°C)	DHPLC analysis	
					Temp of elution (°C)	Buffer (%)
2	AGGTAAGATTGGTACTAGAG	AATAACTAAGTTCATTGCTC	350	58	55	57
					57	57
3	TGAGAAGCAATGTCAGTATAG	CCTGTACATGCCACACGC	201	58	55	53
					53	53
4	CTGGCTTCTGCTACTCTG	TCCCACTTCTCCACACTC	242	58	54	53
					53	53
5	AGTGTTCTTGAATCTTTG	GGCAAATGTGCATATGGC	244	54	57	53
					53	55
6	CTCTGATTGGATGAATTATTC	TTCTTAAAGACAGTAACATGTG	303	59	54	55
					56	55
7	TTTATCTTGACACTCCAGA	ACTCCTCCAGCAATCAATT	237	56	53	53
					53	54
8	GCCCATCGGAGAACAGTCATTAC	GCAAATGACAATAGAATCAGCAG	280	55	56	54
					57	54
9	TTATTCACCTTGTTCTGATGAT	CAAATACTGCAGTATTGATTCC	410	54	54	58
					56	58
10	TATGAATTTGACTGGGATTGG	CTATGGTCACATGTAGACAC	275	59	57	54
					53	56
11	TTGATATTCTGCAATGACTGTG	AGCCACCTCTCCACCTAC	333	62	55	56
					55	56
12_a	TTGTGTGTCAGCTATTGAGG	GGTCTGCTGAGATCTGCTG	406	60	56	60
					59	60
12_b	CAACAACATACCACACCTTC	TTCTCGTGTACTGTGTCTG	422	60	60	60
					60	60
12_c	ACTCCAAGTCTGTGAGCAAC	AGATGGACCCTCATCACATC	541	60	57	63
					57	55
13	GGTATGGTCTAGTCTAC	CACTTCAACTTATTTGTGGG	298	60	59	55
					59	55
14	CAATAGAGTGAGACCCTGTC	CTGAGTCGGTGAAAGCAGTG	279	65	57	55
					60	55
15	AAAAGTCCATCAGTGACTTAC	CCTAGCAGGAGAAAGGACAC	262	60	56	54
					56	54
16	TATAGGAACCTAGTGTCAATGC	CAACTTTGATTGCCAAGTGC	293	59	53	57
					53	55
17	CTGGGTGTGGTTGCATATC	CTGTAACATTGAGAGGGCTAAG	296	60	59	55
					59	55
18	CTTGACATGCTTGCCCTTC	CACCCAGCTGTTCAGAGTAG	418	62	61	58
					61	58
19	ACTCTGGTCAATGGGATGTG	CATTCAAGTCTAGGGTCTG	249	60	59	53
					59	53
20	TTGGCTTCAGCTGGTGTCTG	CATCTGCATTCTACAGCTC	345	61	61	58
					61	58
21	CATTAGCCAGAGTGCACCTG	AGGAAAACTCAACCTCAGCG	290	60	59	55
					63	54

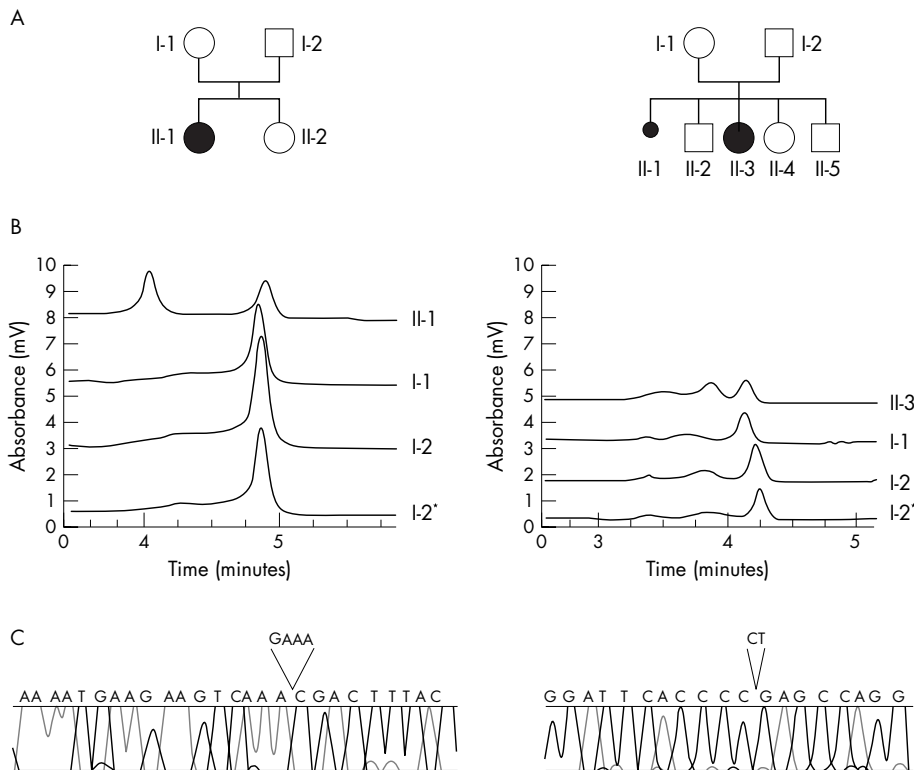


Figure 1 Pedigree (A), DHPLC analysis (B), and DNA sequencing (C). Left side, case 1, right side, case 2. (B) An asterisk indicates that the DNA of the father is 1:1 mixed with a control male. (C) The chromatograms represent the mutated sequences separated from normal alleles on 6% polyacrylamide gel. Deleted bases are indicated above.

Patient 2

This patient is a girl, now aged 8 years. Her pedigree is represented in fig 1A (right). The mother had a normal pregnancy and delivery, and the child was affected with slight cyanosis at birth. Her birth weight was 3800 g but no data concerning her head circumference are available. At 10 days, generalised convulsions, lasting only a few seconds, appeared, which were difficult to control with various antiepileptic drugs and persisted during the following years. Myoclonic seizures, when her arms suddenly opened, possibly infantile spasms, were also reported by her parents, although clinical records concerning these details were not available. Her MRI was normal. Her development was delayed; in the first years of life she did not respond to interactions, she had been found hypotonic by medical professionals, and she was unable to hold an object in her hands. She was able to sit alone at 1.5 years of age. She was examined in our unit when 4 years 10 months of age. Her head circumference was 49 cm (tenth centile), she had a moderate degree of generalised hypotonus, and was unable to hold an object in her hands, which were brought frequently to her mouth and occasionally twisted together. Furthermore, she had myoclonic epileptic fits occurring two or three times a day, and occasional generalised convulsions. An EEG showed generalised discharges of sharp and slow waves and focal sharp waves in the right centrottemporal region. She was evaluated at the age of 7 years 10 months, when a slight improvement in the use of her hands was noted. She could hold a biscuit and some bread in her hands and eat them. Her facial expression varied notably and she was capable of reciprocal modulations. Generalised hypotonus was still present: she was able to sit unaided but unable to walk without support. The stereotypic activities of her hands were still present. She occasionally uttered one or two words. Her head circumference was 50 cm (tenth centile). Scoliosis, kyphosis, cold extremities, and constipation were absent. Myoclonic fits and generalised convulsions were still

present in spite of various treatments, including phenobarbital, valproate, lamotrigine, and carbamazepine. An EEG showed the persistence of generalised sharp and slow waves and the presence of additional multifocal abnormalities.

Molecular analysis

MECP2 point mutations, including the recently identified exon 1, and gross rearrangements were excluded by DHPLC and quantitative PCR, respectively.²⁰⁻²² Based on the partial clinical overlap between the Hanefeld variant and ISSX, we screened the two RTT variant patients for point mutations in *ARX* and *CDKL5*. *ARX* analysis did not identify any mutation. DHPLC analysis of *CDKL5* revealed a different frameshift mutation in the two patients, absent in the DNA of the parents in both cases (fig 1B).

In case 1 we identified a 4 bp deletion in exon 5 (c.163_166delGAAA) (fig 1C; left) leading to loss of most of the *CDKL5* protein (NP_003150). The frameshift mutation creates a stop codon in position 74, after a short stretch of abnormal amino acids (fig 2A, 2B; top). This deletion interrupts the catalytic domain covering the first 300 amino acids, and creates a non-functional *CDKL5* product.

Case 2 showed a 2 bp deletion in exon 18 (c.2635_2636delCT) (fig 1C; right) leading to protein truncation in position 908, after a short stretch of incorrect amino acids (fig 2A, 2B; bottom). This deletion eliminates a putative signal peptidase I serine active site, as predicted by the ScanProsite program (<http://ca.expasy.org/prosite>) (fig 2A).

We then investigated whether mutated and normal alleles were expressed in the two patients. We studied X inactivation in DNA from blood cells of both patients. The assay showed balanced X inactivation in both cases (not shown). Band intensities were measured: the ratio between the two alleles was 52:48 in case 1 and 67:33 in case 2. We then performed RT-PCR to test if mutated mRNA alleles were degraded by nonsense mediated RNA decay. RT-PCR products, separated by electrophoresis and silver stained, demonstrated the

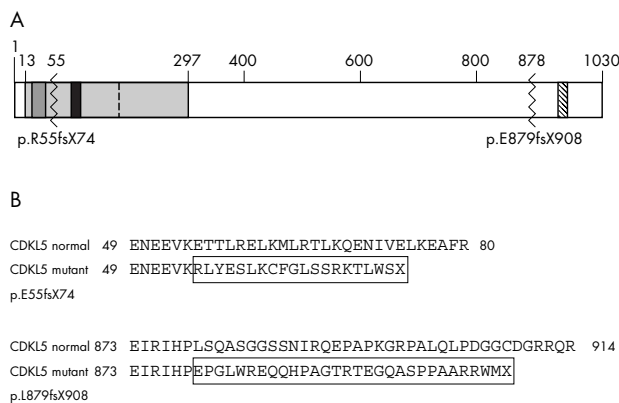


Figure 2 CDKL5 protein with mutation positions (A) and alignment between the normal and the mutated amino acid sequences (B). (A) The catalytic domain (light grey) contains an ATP binding site (dark grey) and the serine–threonine protein kinase active site (dark box). The conserved Thr-Xaa-Tyr motif is indicated with a sketch line. The signal peptidase I serine active site is represented by the reticulated box. The two frameshift deletions are indicated by zigzag lines. The numbers at the top refer to the amino acid positions. (B) In patient 1 (top), the deletion creates a stop codon in position 74. In patient 2 (bottom), the deletion leads to protein truncation in position 908. Stretches of incorrect amino acids are boxed.

expression of both mutated and normal alleles in the two patients (fig 3).

We then extended the *CDKL5* analysis to 19 classic RTT and 15 PSV cases, all *MECP2* negative; no mutation was found.

DISCUSSION

The two girls reported above had a similar clinical course: they showed convulsions very early in life, respectively at 1.5 months and at 10 days, drug resistant in both cases. They otherwise fulfilled the criteria for RTT, including acquired microcephaly, hand apraxia, generalised hypotonus, and stereotypic hand activities.^{4 5 16} Hyperventilation was present in one girl. Scoliosis, constipation, and cold feet were absent in both, suggesting a possibly better preserved autonomic system. It was difficult to retrospectively assess whether they had had the typical stage sequence of RTT. When examined at ages close to 8 and 5 years, they were expressive and interacted in a manner adequate to their reduced general abilities, and they fulfilled the criteria for the early seizure variant of RTT.^{4 5 16} It should be noted that only a few cases of this disorder have been reported^{4 5} and, with the exception of the first case described by Hanefeld (personal communication), no *MECP2* mutations have been described. In the two

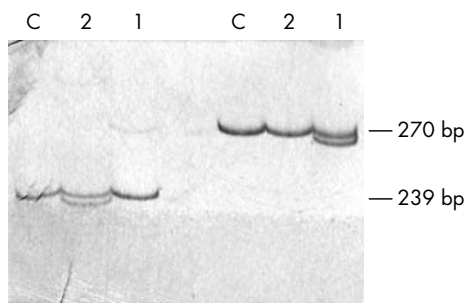


Figure 3 RT-PCR analysis of patients (1 and 2) and a control individual (C). The polyacrylamide gel shows RT-PCR products. RT-PCR products spanning exons 17–18 (left) and exons 4–6 (right). In both cases, the mutant allele is evident as a lower band. Size is indicated on the right.

RTT variant patients, *MECP2* point mutations and gross rearrangements were excluded by DHPLC and qPCR.

Our results indicate that RTT variant with infantile spasms may be due to inactivating *CDKL5* mutations. *CDKL5* is a member of the serine–threonine kinase gene family.²³ Kinase proteins are a large superfamily of homologous proteins, characterised by a highly conserved kinase domain (250–300 amino acids). The *CDKL5* kinase domain is most closely related to human *KKIALRE* and *KKIAMRE* and their orthologues.²³ The sequence alignment of the *CDKL5* protein with these homologues showed two kinase signatures in the catalytic domain: an ATP binding region (amino acids 14 to 47) and a serine–threonine protein kinase active site (amino acids 127 to 144).²³ In addition, a Thr-Xaa-Tyr motif was identified,²³ and the dual phosphorylation of these Thr and Tyr residues has been shown to be essential for activation of the MAP kinase group.²⁴

The frameshift mutation found in patient 1 is located in the conserved kinase domain of the *CDKL5* protein, causing loss of both the serine threonine active site and the Thr-Xaa-Tyr motif. Thus, the deletion abolishes the catalytic function of the protein. Furthermore, it has been reported that lack of functional *CDKL5* protein causes severe ISSX.¹⁵ This finding was derived from a study of two severely affected ISSX female patients with apparently de novo balanced X;autosome translocations, both disrupting the *CDKL5* gene in the kinase domain. Additional studies are needed to further elucidate why different truncating mutations in *CDKL5* cause different but overlapping phenotypes.

Except for the kinase domain, the function of the *CDKL5* protein is unknown (fig 2A). The region between positions 300 and 1030 is not conserved in different species and does not share a homology with other human proteins, making it difficult to predict its function.²⁵ The deletion found in case 2 falls in the C terminus of the protein. We could speculate that this region contains a functional domain specific for human *CDKL5*. Using the ScanProsite program we identified a signal peptidase I serine active site (GTSMCPTL), located between positions 971 and 978, abolished by the deletion in patient 2. This domain is one of the five conserved domains present in all known signal peptidases.²⁶ *CDKL5* probably does not have a peptidase activity, as it lacks other conserved residues necessary for the catalytic activity.^{26 27} However, we cannot exclude that *CDKL5* might form a complex with other proteins that harbour the other essential domains.

A C terminal deletion of *CDKL5* has already been reported by Huopaniemi *et al*²⁸ in a family with X linked juvenile retinoschisis (RS). No additional phenotype was reported in these patients with the exception of epilepsy in one. This deletion, spanning from the 5' region of the RS1 gene to intron 3, disrupts two other genes, *PPEF-1* and *CDKL5* (exon 20). It is possible that this truncation, more 3' than that reported here, generates a milder phenotype.

Finally, it is important to understand why *MECP2* and *CDKL5* mutations lead to a similar phenotype. *MeCP2* and *CDKL5* could belong to the same signalling pathway. As it has demonstrated that *MeCP2* is subjected to phosphorylation and that *CDKL5* has a kinase domain, it is possible that *MeCP2* is directly phosphorylated by *CDKL5*.^{23 29} However, at least in patient 1, *CDKL5* kinase activity is abolished, and a reduced degree of *MeCP2* phosphorylation would lead to a reduction in its dissociation from methylated DNA and to a gene silencing increase.²⁹ However, is known that *MECP2* mutations presumably cause a reduction of gene silencing.^{9 30} Alternatively, *CDKL5* might phosphorylate a second protein that could dephosphorylate *MeCP2*. Additional studies are necessary to determine whether *MeCP2*-*CDKL5* interaction really exists and to unravel the complex mechanisms underlying the above phenotypes.

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