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

E Scala, F Ariani, F Mari, R Caselli, C Pesucchini, I Longo, I Meloni, D Giachino, M Bruttini, G Hayek, M Zappella, A Renieri

**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.

**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 analyser (PE Applied Biosystems, Foster City, CA, USA). PCR products resulting in abnormal DHPLC profiles were preferential allele amplification. RNA isolation from lymphoblasts and cDNA synthesis were performed according to standard protocols. We used primers designed to form cDNA products spanning exons 4–6 (4RTf: 5′-GAAGAGCATGAAATTGTGGCG-3′; 6RTr: 5′-GTGAATAGCCTTGATTAGCTG-3′) and 17–18 (17RTf: GAGAAGATCCTACGACTGCG; 18RTr: AGCTGGAGGGCTGCGTG). RT-PCR products were separated by electrophoresis through a 6% polyacrylamide gel and silver stained.

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

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<tr>
<th>Exon</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product length (bp)</th>
<th>PCR annealing temp (°C)</th>
<th>DHPLC analysis</th>
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<td>Temp of elution (°C)</td>
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**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.
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**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. She was brought moderately often 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 centrotemporal 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. 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 the 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 existence of both normal and mutated alleles.
The first case described by Hanefeld (personal communication) was a variant of RTT. It should be noted that only a few cases of RTT have been reported, and they fulfilled the criteria for the early seizure syndrome. At ages close to 8 and 5 years, they were expressive and communicative, and they interacted in a manner adequate to their reduced general abilities. However, 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 mental retardation. No additional phenotype was reported in these patients with the exception of epilepsy in one. This epilepsy is a well-known feature of RTT. Eighty percent of patients with RTT have at least one seizure type in childhood, and half of them have myoclonic and/or tonic seizures.

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 mental retardation. No additional phenotype was reported in these patients with the exception of epilepsy in one. This epilepsy is a well-known feature of RTT. Eighty percent of patients with RTT have at least one seizure type in childhood, and half of them have myoclonic and/or tonic seizures.

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. 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. The deletion found in case 2 really exists and to unravel the complex mechanisms underlying ISSX is an important goal for further research. The deletion found in case 2 is located in the conserved kinase domain of the CDKL5 protein. CDKL5 is a serine–threonine kinase that is highly conserved in different species and does not share a homology with other human proteins, making it difficult to predict its function. The deletion found in case 2 really exists and to unravel the complex mechanisms underlying ISSX is an important goal for further research.

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 (amino acids 1 to 14). 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.

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 1–4 and exons 6–9 are shown. The two frameshift deletions are indicated by zigzag lines. The numbers at the top refer to the amino acid positions.
AKNOWLEDGEMENTS

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REFERENCES

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