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

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

Background: 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). 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. Approximately 80% of patients with classic RTT have a mutation in the methyl CpG binding protein 2 gene (MECP2; OMIM #300005). MECP2 mutations have also been identified in about 50% of PSV cases and in a lower percentage of other variants. In the variant with early development of convulsion described by Hanefeld, MECP2 mutations have not been published.

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 aristaeless related homeobox (ARX) gene, which maps to Xp21.3–p22.1. 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. Recently, a second gene has been found to be involved in ISSX, the cyclin dependent kinase-like 5 gene (CDKL5/STK9; NM_003159). These authors characterised two unrelated female patients with an apparently balanced translocation, 46,XX,t(X;7)(p22.3;p15) in one case and 46,XX,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.

We then investigated 19 classic RTT and 15 PSV patients. The girls with classic RTT were diagnosed according to the international criteria. The PSV girls fulfilled the criteria of Hagberg and Skjedal for RTT variants. In particular, the PSV girls were diagnosed according to the international criteria.
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 silver stained. Bands were measured using the Diversity Database analyser (PE Applied Biosystems, Foster City, CA, USA). PCR fluorescencing terminators on an ABI Prism 310 genetic analyser (PE Applied Biosystems, Foster City, CA, USA). 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 handwashing 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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**Table 1** Primer sequences and conditions for PCR reaction and DHPLC analysis of **CDKL5** amplicons
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Absence of myoclonic fits and generalised convulsions were still present. She occasionally uttered one or two words. Her head circumference was 50 cm (tenth centile), she was able to sit unaided but unable to hold an object in her hands. She was able to sit alone at 1.5 years of age. She was delayed; in the first years of life she did not respond to professionals, and she was unable to hold an object in her hands. She was capable of reciprocal modulations. Generalised hypotonia, and was 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. 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 presence of splicing.
MECP2: the first case described by Hanefeld (personal communications, and they fulfilled the criteria for the early seizure system. It was difficult to retrospectively assess whether they had had the typical stage sequence of RTT. When examined in one girl. Scoliosis, constipation, and cold feet were absent otherwise fulfilled the criteria for RTT, including acquired stereotypic hand activities. Hyperventilation was present they showed convulsions very early in life, respectively at ages close to 8 and 5 years, they were expressive and had had the typical stage sequence of RTT. When examined

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. 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. It should be noted that only a few cases of this disorder have been reported and, with the exception of the first case described by Hanefeld (personal communications), no MECP2 mutations have been described. In the two 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. 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. 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). In addition, a Thr-Xaa-Tyr motif was identified, and the dual phosphorylation of these Thr and Tyr residues has been shown to be essential for activation of the MAP kinase group.

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 Xautosome 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. 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 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 peptides. CDKL5 probably does not have a peptidase activity, as it lacks other conserved residues necessary for the catalytic activity. 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 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. 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. However, is known that MECP2 mutations presumably cause a reduction of gene silencing. 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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Authors’ affiliations
E Scala, F Ariani, F Mari, R Caselli, C Pescucci, I Longo, I Meloni, D Giachino, M Bruttini, A Renieri, Medical Genetics, Department of Molecular Biology, University of Siena
G Hayek, M Zappella, Child Neuropsychiatry, University of Siena
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REFERENCES