Original Article

Novel *CDKL5* Mutations in Czech Patients with Phenotypes of Atypical Rett Syndrome and Early-Onset Epileptic Encephalopathy

(*CDKL5* / cyclin-dependent kinase-like 5 protein / early-onset epileptic encephalopathy / early-onset seizure variant of Rett syndrome)

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Abstract. The X-linked *CDKL5* gene, which encodes cyclin-dependent kinase-like 5 protein, has been implicated in early-onset encephalopathy and atypical Rett syndrome with early-onset seizures. The CDKL5 protein is a kinase required for neuronal development and morphogenesis, but its precise functions are still largely unexplored. Individuals with *CDKL5* mutations present with severe global developmental delay, intractable epilepsy, and Rett-like features. A clear genotype-phenotype correlation has not been established due to an insufficient number of reported cases. The aim of this study was to analyse the *CDKL5* gene in Czech patients with early-onset seizures and Rett-like features. We performed mutation screening in a cohort of 83 individuals using high-resolution melting analysis, DNA sequencing and multiplex ligation-dependent probe amplification. Molecular analyses revealed heterozygous pathogenic mutations in three girls with severe intellectual disability and intractable epilepsy starting at the age of two months. All three identified mutations, c.637G>A, c.902_977+29del105, and c.1757_1758delCT, are novel, thus significantly extending the growing spectrum of known pathogenic *CDKL5* sequence variants. Our results support the importance of genetic testing of the *CDKL5* gene in patients with early-onset epileptic encephalopathy and Rett-like features with early-onset seizures. This is the first study referring to molecular defects of *CDKL5* in Czech cases.

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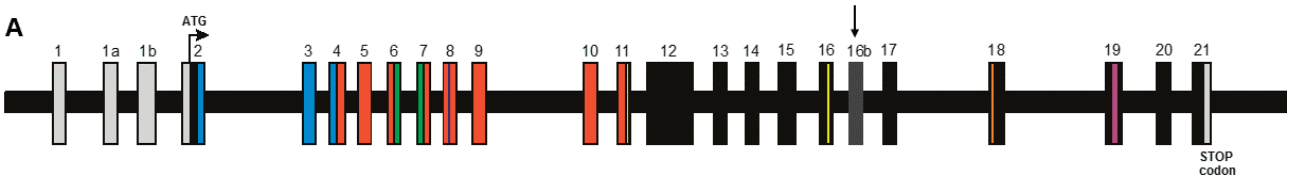
Abbreviations: ATP– adenosine triphosphate, CDKL5 – cyclin-dependent kinase-like 5 protein, EEG – electroencephalogram, HGMD – human gene mutation database, HRM – high-resolution melting, IVF – *in vitro* fertilization, MeCP2 – methyl-CpG-binding protein 2, MAP – mitogen-activated protein, MLPA – multiplex ligation-dependent probe amplification, NES – nuclear export signal, NLS – nuclear localization signal, PCR – polymerase chain reaction, RTT – Rett syndrome, XCI – X-chromosome inactivation.

Introduction

Mutations in the X-linked *CDKL5* gene (OMIM #300203) were first identified in patients with early infantile epileptic encephalopathy 2 (Kalscheuer et al., 2003) and later in patients with early-onset seizures and symptoms resembling Rett syndrome (RTT) (Tao et al., 2004; Weaving et al., 2004). RTT is a severe, X-linked dominant, neurodevelopmental disorder primarily caused by *de novo* mutations in the methyl-CpG-binding protein 2 gene (*MECP2*). RTT is characterized by developmental regression, loss of speech and motor functions, stereotypic hand movements, and autistic features. Epileptic seizures are a very common additional feature of RTT (Amir et al., 1999). A growing number of described cases with *CDKL5* mutations had allowed

Fig. 1. Schematic structure of the *CDKL5* gene (**A**) and the main isoform (1030 aa) of the CDKL5 protein (**B**)



Exons 1, 1a, and 1b are untranslated. The translation initiation codon ATG lies in exon 2. Alternatively spliced exon 16b is shown by an arrow.

Functional domains and motifs of the CDKL5 protein. NLS: nuclear localization signal, NES: nuclear export signal

better characterization of associated clinical symptoms, and the term *CDKL5* disorder was proposed (Fehr et al., 2012). Girls and boys with the *CDKL5* disorder show severe global developmental delay and epilepsy with an onset within the first months of life. Rett-like features, including acquired microcephaly, stereotypic hand movements, poor eye contact, and poor or no speech and purposeful hand use, are also very common (Bahi-Buisson and Bienvenu, 2012).

The human *CDKL5* gene is located on chromosome Xp22 and consists of 24 exons, of which the first three are untranslated (Fig. 1A). The gene encodes the cyclin-dependent kinase-like 5 protein (CDKL5), which belongs to the CMGC family of serine/threonine kinases. There are at least four CDKL5 isoforms characterized by the alternative splicing of exon 16b and an altered C-terminal region (Mari et al., 2005; Fichou et al., 2011; Rademacher et al., 2011; Williamson et al., 2012). The *CDKL5* gene is expressed in a wide variety of tissues, especially in the brain. Moreover, the isoforms containing exon 16b are specifically found in the brain (Fichou et al., 2011). The N-terminal catalytic domain contains the ATP-binding region, the serine/threonine protein kinase active site, and the auto-phosphorylating Thr-Xaa-Tyr motif. The signals for nuclear import, nuclear export, and the putative signal peptidase I serine active site are located in the long C-terminal region (Fig. 1B). CDKL5 shuttles between the nucleus and the cytoplasm, indicating its roles in both cellular compartments (Rusconi et al., 2008). The precise functions of CDKL5 are still not fully understood, but available clinical and molecular data, including expression profiles, clearly suggest its involvement in brain development and neuronal maturation. CDKL5 participates in the regulation of actin cytoskeleton and dendritic arborization (Chen et al., 2010). It has also been proposed to phosphorylate the epigenetic factor MeCP2 (Lin et al., 2005; Mari et al., 2005) and DNA methyltransferase 1 (Kameshita et al., 2008), thereby participating in the regulation of gene expression and DNA methylation. The overlapping clinical features observed in individuals with *CDKL5* disorder and RTT strengthen the suggestion of a functional relationship between CDKL5 and MeCP2. Common pathways may be affected when either protein is deficient.

To date, more than 200 mutations have been identified in the *CDKL5* gene (HGMD Professional 2015.3 (Stenson et al., 2014)), including missense and nonsense mutations, splice site mutations, small to large deletions, and duplications. Thus far, no definitive correlation between clinical presentations and specific mutations has been established. However, patients with mutations in the C-terminal region present with milder clinical symptoms than patients with mutations in the N-terminal catalytic domain, and males are generally more severely affected than females (Fehr et al., 2015). Pathogenic *CDKL5* mutations arise *de novo* and most patients are sporadic cases. Familial occurrence is extremely rare and likely caused by germline mosaicism (Weaving et al., 2004; Hagebeuk et al., 2015). Several *CDKL5* variants inherited from either asymptomatic mother or father have been reported, but these are described by authors as likely non-pathogenic or only potentially pathogenic (Nectoux et al., 2006; Rosas-Vargas et al., 2008; Schaaf et al., 2011; Diebold et al., 2014).

The *CDKL5* gene is subject to X-chromosome inactivation (XCI), hence the clinical severity in female patients with a *CDKL5* mutation may be due to a variable XCI pattern. However, XCI in peripheral blood, which is the most routinely studied material, may not necessarily reflect the XCI pattern in the brain. It is also important to take into account the possible region to region differences of the XCI in the brain (Sharp et al., 2000).

This is the first study referring to *CDKL5* mutations in Czech patients. We performed the mutation screening in a cohort of 83 patients with Rett-like features and early-onset epileptic encephalopathy and describe three novel pathogenic mutations.

Material and Methods

Patients

The patients were recruited from several departments of child neurology and clinical genetics in the Czech Republic. Thirty girls presented with atypical RTT or Rett-like features with early-onset seizures and previously tested negative for *MECP2* mutations. Fifty-four patients (28 girls, 26 boys) exhibited idiopathic epileptic encephalopathy. All eligible patients had their first seizure before the age of 12 months. Genomic DNA was extracted from peripheral blood leukocytes using the salting out procedure. Alternatively, other hospitals collected, extracted and then shipped patient DNA samples.

This study received approval from the Committee of Medical Ethics at the First Faculty of Medicine, Charles University in Prague, and written informed consent was obtained from the parents of all patients.

High-resolution melting analysis (HRM)

The coding exons 2–21 (including exon 16a and excluding exons 5, 15, and 21) with flanking non-coding regions were amplified in a total volume of 10 µl containing 1× Plain Combi PP Master Mix (Top Bio, Prague, Czech Republic), 200 nM of each primer, 2–4% DMSO, 1× LCGreen Plus+ (BioFire Defense, Salt Lake City, UT), and 20 ng of genomic DNA. The PCR cycling conditions were: initial denaturation at 94 °C for 90 s, 40 cycles of denaturation at 94 °C for 30 s, annealing at 55–63 °C for 30 s, and elongation at 72 °C for 30 s, followed by final extension at 72 °C for 5 min. The primer sequences and specific PCR conditions are listed in Table 1. The HRM and melting curve analyses were performed using the LightScanner Instrument and original LightScanner software (BioFire Defense) according to the manufacturer's instructions. The melting curves were normalized using the default curve shift adjustment (0.05) and the analysis was carried out with the "auto group" option at high sensitivity. The male samples were melted twice. After the first HRM analysis, each male sample on the plate was spiked with a wild-type control sample to create heterozygous conditions and analysed again.

DNA sequencing

Exons 5, 15, and 21, and amplicons with aberrant HRM profiles were amplified in a total volume of 12.5 µl containing 1× PPP Master Mix (Top Bio), 200 nM of each primer, and 20 ng of genomic DNA. The cycling profile included initial denaturation at 94 °C for 90 s, 33 cycles of denaturation at 94° C for 30 s, annealing at 50–62 °C for 40 s, and elongation at 72 °C for 30 s, followed by final extension at 72 °C for 5 min. The primer sequences and specific PCR conditions are listed in Table 1. The PCR products were purified, sequenced, and analysed in

Table 1. Primer sequences (5′→3′ direction) and PCR conditions for amplification of CDKL5 exons

Amplicon	Length (bp)	Forward primer	Reverse primer	Ta (ºC) HRM	Ta (ºC) seq
Exon 2	319	gattggttactagagtactgcc	actcaaggaacgcaaaaattcc	63	53
Exon 3	237	aagcaatgtcagtatagcagag	acatttctggtctcaagcat	63	53
Exon 4	241	ctggcttcttgctactctgtc	cccacttcttccacactctatg	63	62
Exon 5	342	tccattcttgtacttcttatg	taaataatgtcagaaacagcc	-	50
Exon 6	267	ctagatgctttgtaaaattg	catgtgaaatactcttaact	55	53
Exon 7	305	ctgccacagttttctattc	tcctctaccaatttaagtca	55	53
Exon 8	298	ataaatagcccatgcgagaa	atgaactcaaagcaaatgac	55	53
Exon 9	360	aagtgtgctgcacataaatttg	tgcagtattgattccattttgg	63	53
Exon 10	263	ttttgcttatctgctccctt	cataaaagcaggctatggtc	55	53
Exon 11	336	attctgcaatgactgtgtatttctt	atttaagcagccacctcctc	59	62
Exon 12.1	313	gaaggcacatgcttgagaga	cttggtaggttttggtgtgc	59	62
Exon 12.2	325	aaggtctccctgccaatgaa	cgactctgcttttcattggg	63	62
Exon 12.3	345	ccagggacaaagtacctcaa	gctgagcaaagttctcgtgt	59	62
Exon 12.4	275	accagtaggtacttcccatc	gaagaaaaggggctggtgta	63	62
Exon 12.5	278	cagtagccattcccattcac	tcatcacatccctcgtacca	63	53
exon 13	254	gctggttatggtcctagttc	ctaggcagtaatgatgttga	59	53
Exon 14	333	gggcagtcaaggctacagtaa	cctgagtcggtgaaagcagt	59	62
Exon 15	522	gttatgtgaagagctgatga	actaaaaagctcatccagaa	-	50
Exon 16	240	ttatatttgtcacacaatgg	gcaaagtgtaaagtatccat	59	53
Exon 16b	192	ttgcaattagtttatttgtatctt	gcagttttcagggctaccata	55	53
Exon 17	255	gggtgtggttgcatatctta	tccccgctcctcaggacagtta	63	62
Exon 18	332	ttccctccccagccttatggtc	tgtggcactcctggtcacagag	63	62
Exon 19	216	aatatgtttgtgttcttaaagg	agtttcattcagtagtctaggg	59	53
Exon 20	322	tggtgtctgggagccgat	tacagctcctcgggcaattc	63	62
Exon 21	426	atgtggccttctgctccgtg	ctcaggcaatgcacctgctc	-	62

ABI PRISM 3100-Avant and 3500xL genetic analysers (Applied Biosystems, Foster City, CA). The acquired sequences were compared with the GenBank Reference sequence NG_008475.

Multiplex ligation-dependent probe amplification (MLPA)

All samples were tested for large deletions using the SALSA MLPA P189 probe mix (MRC-Holland, Amsterdam, Netherlands) according to the manufacturer's instructions. Fragment analysis was performed in ABI PRISM 3100-Avant and 3500xL genetic analysers (Applied Biosystems), and the MLPA data were analysed using Coffalyser.Net software (MRC-Holland).

X-chromosome inactivation (XCI)

The XCI pattern was determined using a modified HUMARA assay (Allen et al., 1992). The forward primer was fluorescently labelled with 6-FAM, and the PCR products were resolved through capillary electrophoresis in a 3500xL genetic analyser (Applied Biosystems). The data were analysed using GeneMarker software (Softgenetics, State College, PA). The XCI was considered to be skewed if the ratio between active and inactive alleles was higher than 75 %.

Results

In this study, we analysed the *CDKL5* gene in 83 Czech patients with Rett-like features and early-onset seizures (30 girls) and early-onset epileptic encephalopathy (27 girls, 26 boys). Molecular analyses revealed 15 sequence variants, including three novel heterozygous pathogenic mutations (Table 2). Most sequence variants were initially discovered by HRM. Once an abnormal melting profile was detected, sequencing of the corresponding amplicon was carried out to identify the specific sequence change. Polymorphisms located in exons 5, 15, and 21 were found directly by DNA sequencing because these exons did not undergo the HRM analysis. We report two novel deletions, c.902_977+29del105 (p.Arg301Lysfs*24), involving parts of exon 11 and intron 11, and c.1757_1758delCT (p.Ser586Cysfs*24) located in exon 12 (Fig. 2). The mutations cause a frameshift and premature translation termination, unless the mutated transcripts are eliminated by nonsense-mediated mRNA decay. The novel missense mutation c.637G>A (p.Gly213Arg) is localized in exon 9 (Fig. 2). The possible damaging impact of amino acid substitution on the protein structure and function was investigated using several prediction tools with the following results:

1) SIFT (http://sift.jcvi.org/), damaging (score 0);
2) PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/), probably damaging (score 1);
3) Mutation Taster (http://www.mutationtaster.org/), disease-causing;
4) SNPs&GO (http://snps.biofold.org/snps-and-go/snps-and-go.html), disease-causing (score 0.752);
5) PredictSNP (http://loschmidt.chemi.muni.cz/predictsnp/), deleterious (expected accuracy 87 %);
6) PMut (http://mmb.pcb.ub.es/PMut/), neutral (score 0.42);
7) MutPred (http://mutpred.mutdb.org/), pathological (score 0.801).

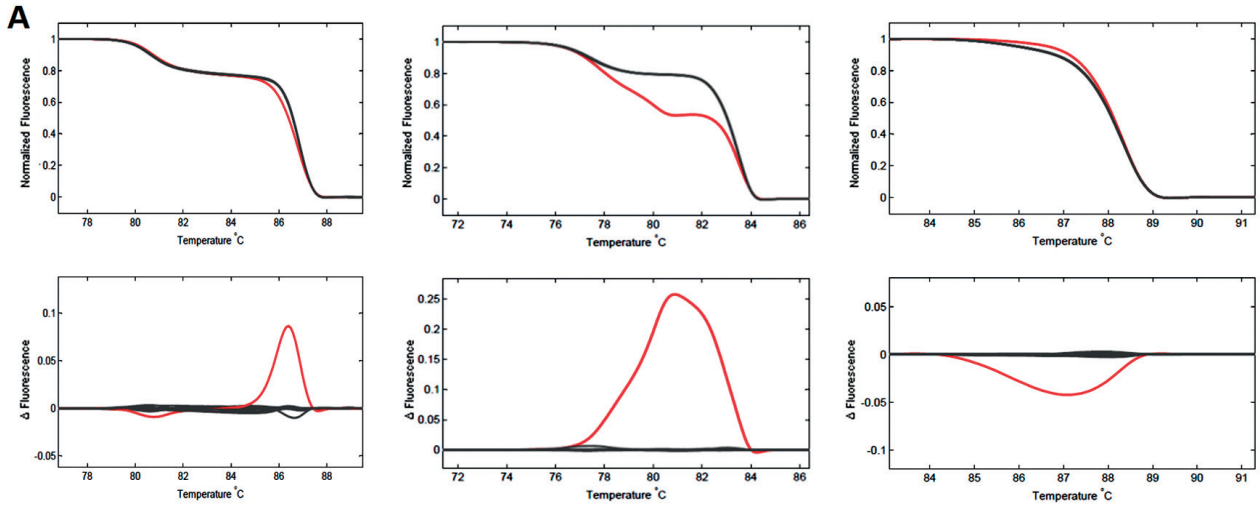
The identified mutations were tested in the parents of the respective patients and 200 control samples. All tested samples were negative for the mutations. The biological mother of the girl with the c.637G>A mutation was not available for testing as she is an anonymous oocyte donor. The mutation was not present in the pa-

Table 2. CDKL5 variants identified in a Czech cohort

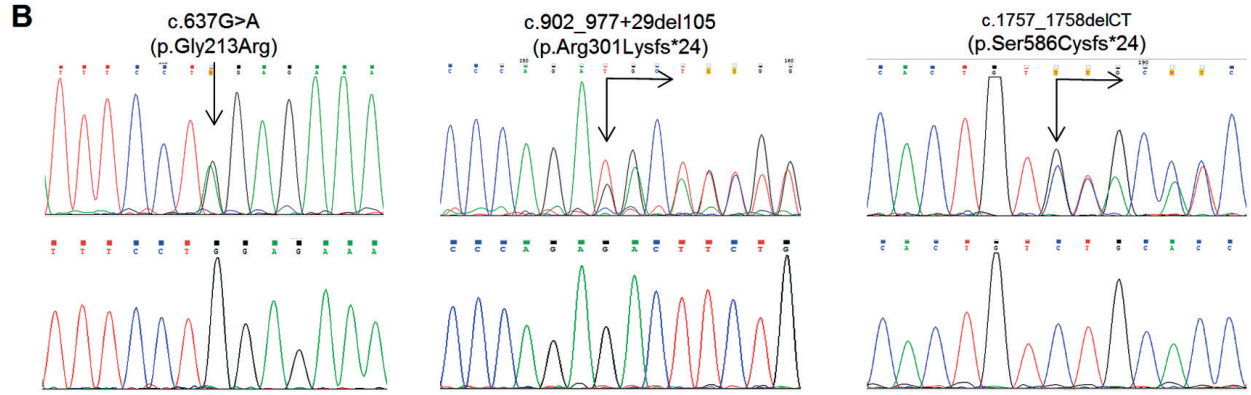
Location	Nucleotide change	Protein change	Domain	Cases (gender)
Intron 3	c.100-73C>T	-	-	1 (M)
Intron 4	c.145+17A>G	-	-	2 (M)
Intron 4	c.146-98A>G	-	-	2 (M)
Intron 6	c.404-57T>C	-	-	2 (M)
Exon 9	**c.637G>A**	**p.Gly213Arg**	**catalytic**	**1 (F)**
Exon 11	**c.902_977+29del105**	**p.Arg301Lysfs*24**	**C-terminal**	**1 (F)**
Exon 11	c.915T>C	p.Arg305=	C-terminal	1 (F)
Exon 12	**c.1757_1758delCT**	**p.Ser586Cysfs*24**	**C-terminal**	**1 (F)**
Intron 14	c.2153-47_2153-38del10	-	-	2 (M)
Exon 16	c.2372A>C	p.Gln791Pro	C-terminal	2 (M), 2 (F)
Exon 18	c.2541G>A	p.Ser847=	C-terminal	1 (F)
Exon 21	c.3003C>T	p.His1001=	C-terminal	2 (M)
Exon 21	c.3084G>A	p.Thr1028=	C-terminal	2 (M)
3´UTR	c.*131A>G	-	-	35 (F), 15 (M)
3´UTR	c.*132T>A	-	-	32 (F), 12 (M)

Numbering is based on the GenBank reference sequence NM_003159. Pathogenic mutations (all novel) are highlighted in bold. M – male, F – female

Fig. 2. Novel *CDKL5* mutations identified in Czech patients



HRM curves showing normalized melting curves (top) and difference plots (bottom). Red curves represent the melting profiles of the PCR products that contain the mutations, and grey curves represent the wild-type samples.



Sequencing analysis of the amplicons with an aberrant HRM profile confirming the presence of the mutation (top). Control sequence to the corresponding region (bottom)

tient's healthy twin sister; nevertheless, its *de novo* origin could not be definitively confirmed. Additionally, none of the novel mutations were found in SNP databases (dbSNP and 1000Genomes), confirming that they are rare sequence variants.

The XCI patterns of the patients with confirmed *CDKL5* mutations were random; the influence of the XCI on the severity of clinical phenotype could thus not be evaluated.

Discussion

Two main clinical presentations associated with mutations in the *CDKL5* gene include early-onset epileptic encephalopathy and Rett-like features with early-onset seizures. Although some genotype-phenotype associations have been observed, a clear correlation has not yet been confirmed. More individuals with *CDKL5* mutations need to be evaluated to further investigate the role of the genotype on clinical severity and to develop appropriate prognostic information for clinicians. We performed molecular analyses in a cohort of 83 Czech patients and describe clinical and molecular aspects in three girls with novel *CDKL5* mutations.

The patient carrying the mutation c.1757_1758delCT (p.Ser586Cysfs*24) is a 13-year-old girl with epileptic encephalopathy (Lennox-Gastaut type). The identified mutation, in case of mRNA translation, would lead to synthesis of a truncated CDKL5 protein lacking a part of the C-terminal region. Due to an absent nuclear localization signal, nuclear export signal, and signal peptidase I serine active site, which are located in the C-terminal region of the CDKL5 protein, enhanced kinase activity and aberrant subcellular localization of the mutated protein could be expected (Rusconi et al., 2008). The patient was born from an uneventful pregnancy, and there were no concerns during the first two months of life. After the first two months of life, the patient became placid and sleepy. The first seizures occurred at the age of five months, alternating between generalized tonic and tonic-clonic seizures. Her electroencephalogram (EEG) showed multifocal spikes, and

the antiepileptic treatment did not significantly reduce the frequency of the seizures. At the age of two years, flexion spasms were replaced by extension spasms. The girl presents with severe intellectual disability with autistic features, no speech, and poor hand use. Stereotypic hand movements have not been reported and she suffers from microcephaly (below the 3rd percentile). She has never been able to walk, has severe scoliosis and is unable to sit unless supported by a corset.

The patient with the c.902_977+29del105 (c.Arg301Lysfs*24) mutation was initially suspected to manifest an early-onset seizure variant of RTT. Before the novel pathogenic mutation in the *CDKL5* gene was identified, the patient tested negative for *MECP2* mutations. The c.902_977+29del105 mutation is located at the beginning of the C-terminal region. The same known functionally relevant regions are affected as in the first case described above. The patient was born at term from an uneventful pregnancy, but her psychomotor development has been delayed since birth. The first tonic seizures occurred at the age of seven weeks, and later infantile spasms presented. Her EEG was initially normal and later showed slow δ waves and specific graphoelements. The patient, now at the age of 15 years, suffers from daily seizures, and the epilepsy remains resistant to several drugs. The current clinical picture is consistent with epileptic encephalopathy (Lennox-Gastaut type). Her head circumference is below the 3rd percentile. She is severely intellectually disabled with autistic features, intermittent eye contact, no speech, and limited hand use. She uses a wheelchair, but can crawl on all fours and even walk a few steps while supported by both hands.

In comparison to the above-mentioned patient carrying the mutation p.Ser586Cysfs*24, the second girl showed earlier onset of developmental delay and seizures, but her overall phenotype was not significantly milder. In the CDKL5 disorder, clinical symptoms, especially the seizures, generally occur within the first months of life, but the precise age at onset tends to vary regardless of the location of mutations. The clinical heterogeneity is likely caused by different molecular and cellular consequences of various *CDKL5* mutations. The C-terminal region of the CDKL5 protein is still rather uncharacterized and the number of patients with the same mutation is limited, which hampers the genotype-phenotype correlation. It was demonstrated that some frameshift and nonsense mutations do not cause a complete absence or significant reduction of mutated transcripts, suggesting that some truncated proteins are produced. Additionally, truncated proteins show different subcellular distribution (Bahi-Buisson et al., 2008b) and therefore, the C-terminal region seems to be responsible for proper subcellular localization of the CDKL5 protein, probably via protein-protein interactions (Bertani et al., 2006; Bahi-Buisson et al., 2008b).

The last novel mutation, c.637G>A (p.Gly213Arg), was identified in a girl with an early-onset seizure variant of RTT and negative *MECP2* test results. The mutation affects a conserved amino acid, glycine 213, in the catalytic domain of the CDKL5 protein. The substitution of the neutral non-polar glycine for the basic positively charged arginine may critically affect the activity of the functional domain. The catalytic domain of the CDKL5 protein is closely related to that of the members of mitogen-activated protein (MAP) kinases, which are known important regulators of synaptic plasticity (Thomas and Huganir, 2004). It appears that missense mutations tend to be localized mainly in the catalytic domain, which confirms the importance of the kinase activity of CDKL5 for the proper brain function and development (Kilstrup-Nielsen et al., 2012). Although the confirmation of the *de novo* origin of the mutation in our patient was not feasible, we determined that the mutation is pathogenic, based on the results of several *in silico* analyses performed with prediction tools. The clinical features of the patient are also consistent with the *CDKL5* disorder.

The patient was born from an uneventful twin pregnancy after IVF with donated oocytes. The delivery was at the 35th week through a C-section due to premature rupture of membranes and the collisional position of the twins. Her early psychomotor development was slightly delayed compared to the twin sister's development. The seizures started at the age of two months and were focal clonic. Currently, the patient dominantly presents with tonic seizures. All seizures occur during sleep. Her EEG was initially normal and later showed slow δ waves or sharp frontal waves during sleep. The EEG is generally poor. Epilepsy remains pharmacoresistant. The patient, currently at the age of three years, presents with severe psychomotor impairment with very slow developmental progression, almost absent purposeful hand use, prominent stereotypic hand movements, bruxism, severe sleep disorder, and dysphagia. Her head circumference is at the 25th percentile, she can sit but is not able to walk, and has no speech (currently uses only two meaningful words). Similar symptoms have been reported in other patients with missense mutations located in the vicinity of glycine 213 (Bahi-Buisson et al., 2008a; Rosas-Vargas et al., 2008; Hagebeuk et al., 2013). However, as mentioned above, the onset of seizures and the severity of phenotype are variable even among the patients with mutations in the same functional domain. Further studies are needed to elucidate the pathogenic effect of different *CDKL5* mutations.

The overlapping phenotypes of patients with *MECP2* and *CDKL5* mutations imply that MeCP2 and CDKL5 proteins play a role in a common pathway. All physiological functions of these proteins are not fully understood, but both proteins interact *in vitro* and *in vivo* (Mari et al., 2005). CDKL5 seems capable of phosphorylating MeCP2 *in vitro*, hence modulating MeCP2 activity and indirectly altering target gene expression (Kilstrup-Nielsen et al., 2012). Impaired CDKL5 activity might, according to this model, influence certain phosphorylation-dependent functions of MeCP2, which would lead to a subset of Rett-like symptoms observed in patients with *CDKL5* mutations. *CDKL5* is also one

of the target genes repressed by MeCP2, which represents another link between the two proteins (Carouge et al., 2010).

In conclusion, mutations in the *CDKL5* gene play an important role in the molecular pathology of epileptic encephalopathies and atypical RTT. All Czech patients with the identified *CDKL5* mutations developed epilepsy by the age of six months, which supports the current opinion that early-onset seizures are an essential clinical feature of the *CDKL5* disorder. Our results emphasize the importance of screening for *CDKL5* mutations, especially in children with severe global developmental delay and early-onset intractable seizures. Defining the phenotypic characteristics of specific *CDKL5* mutations is of great importance because associations between genotype and phenotype are still being elucidated.

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