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DATA REPORT

Recurrent occurrences of *CDKL5* mutations in patients with epileptic encephalopathy

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The *cyclin-dependent kinase-like 5* gene (*CDKL5*) is recognized as one of the genes responsible for epileptic encephalopathy. We identified *CDKL5* mutations in five Japanese patients (one male and four female) with epileptic encephalopathy. Although all mutations were of *de novo* origin, they were located in the same positions as previously reported pathogenic mutations. These recurrent occurrences of *de novo* mutations in the same loci may indicate hot spots of nucleotide alteration.

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Epileptic encephalopathies are characterized by frequent and intractable seizures associated with severe neurological developmental delay.[1] To date, several genetic causes have been revealed, and epileptic encephalopathies are classified into subcategories according to the corresponding genetic alterations identified.[2] At present, an Online Catalog of Human Genes and Genetic Disorders (OMIM database; http://www.omim.org/) lists a total of 23 genes that are associated with the pathogenesis of epileptic encephalopathies.[3]

The *cyclin-dependent kinase-like 5* gene (*CDKL5*), which maps to chromosome Xp22 and contains 20 coding exons, is recognized as one of the genes responsible for a form of epileptic encephalopathy classified as early infantile epileptic encephalopathy 2 in OMIM (EIEE2; MIM#300672). To date, many nucleotide alterations associated with EIEE2 have been identified in *CDKL5*.[4–6] Previously, we reported the results of a cohort study for *CDKL5* mutations involving 12 patients with *CDKL5* alterations.[7] This study reports *CDKL5* mutations identified by genetic diagnosis in five new patients.

This study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of Tokyo Women's Medical University. Thirty-four newly recruited patients were enrolled into this study. All patients were diagnosed as having epileptic encephalopathy on the basis of clinical symptoms and electroencephalography results as described previously.[7] After obtaining written informed consent from the patients' families, blood samples were obtained from the patients and their parents, if necessary. DNA was extracted from these blood samples using a QIAamp DNA extraction kit (Qiagen, Hilden, Germany). Whole-genome copy number was analyzed using the SurePrint G3 Human CGH Microarray Kit 8×60 K (Agilent Technologies, Santa Clara, CA, USA) as described previously,[7] and no aberrations were found. All coding exons of *CDKL5* were analyzed by the standard Sanger sequencing method as described previously.[7]

Patient 1 was a 5-year-old girl. At the age of 1 month, she had suffered from generalized tonic convulsions. These were intractable and occurred daily. These convulsions evolved into a series of spasms. Brain magnetic resonance imaging (MRI) performed at 16 months did not indicate any definitive structural abnormalities (Figures 1a,d,i). Patient 1 was unable to sit unassisted and had poor eye contact, indicating a developmental delay. Sanger sequencing identified XM_005274584.1(CDKL5_v001):c.404-1G>A (Figure 2a). Although this alteration has not been reported previously, a different alteration at the same position affecting a splicing acceptor site, XM_005274584.1:c.404-1G>T, was reported as a pathogenic variant by Archer *et al.*[8] To confirm aberrant splicing, mRNA expression was analyzed by reverse-transcribed-PCR amplification and subsequent Sanger sequencing. In brief, total RNA extracted from the patient's lymphocytes was reverse-transcribed into complementary DNA, which was used as a template for PCR amplification by means of specific primers (sense 5′-CAAATGG AGTTCCACCTGAG-3′ and antisense 5′-AAGGCTGTCCATCGCTAAGC -3′). Normally, a 298 bp product is predicted to be amplified by this reverse-transcribed-PCR. After agarose gel electrophoresis, an abnormal short band was identified (Figure 2b). The normal band and the abnormal short band were separated, and the DNA extracted from these bands was analyzed by Sanger sequencing. The sequencing results of the short band showed a skipping of exon 7 (Figure 2c), which is predicted to cause a 60 bp deletion in the *CDKL5* mRNA (XM_005274584.1:c.404-463del).

Patient 2 was a 19-month-old boy. At the age of 20 days, he experienced tonic convulsions that evolved into intractable epileptic spasms. A brain MRI performed at 19 months revealed no definitive abnormalities (Figures 1e,j). No head control had been achieved to date. Patient 3 was a 4-month-old girl. She started to show tonic convulsions at the age of 20 days. A series of spasms were also observed. An electroencephalography showed multi-focal spikes. A brain MRI performed at 18 weeks demonstrated no definitive abnormality compared with an age-matched control (Figures 1f,k). Patients 2 and 3 had the same mutation, XM_005274584.1:c.533G>A [p.(Arg178Gln)] (Figure 2a). This missense mutation has been recurrently identified and was reported in our previous study[7] and also in a study by Zhao *et al.*[9] A different alteration in the same residue, c.533G>C

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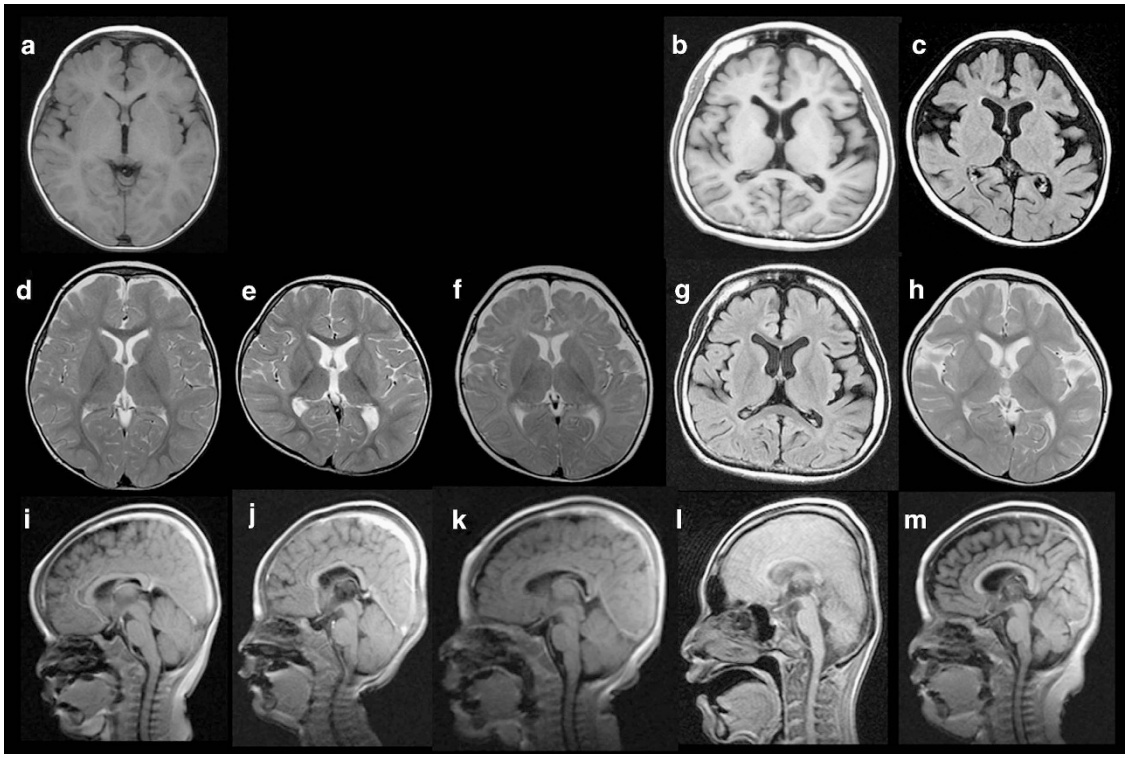

Figure 1. Brain magnetic resonance imaging (MRI) results. (**a**,**b**) T1-weighted axial images; (**c**,**g**) fluid attenuated IR (FLAIR) axial images; (**d**–**f**,**h**) T2-weighted axial images; (**i**–**m**) T1-weighted sagittal images. No definitive abnormality was observed in patient 1 (**a**,**d**,**i**), patient 2 (**e**,**j**), or patient 3 (**f**,**k**). Mild volume loss was observed in patient 4 (**b**,**g**,**l**). Mildly reduced volume was noted, predominantly in the frontal region of patient 5 (**c**,**h**,**m**).

[p.(Arg178Pro)], was also reported by Elia *et al.*[10] and Nemos *et al.*[11]

Patient 4 was a 20-year-old woman. At 2 months of age, she started to show partial seizures associated with vocalization and tonic convulsions, which evolved into intractable epileptic spasms. A brain MRI performed at 20 years of age showed a mildly atrophic change in the brain (Figures 1b,g,l). At present, the patient is unable to sit unassisted or articulate coherent and meaningful words. She was found to have XM_005274584.1:c.825 +1G>A, which affects a splicing donor site (Figure 2a), as previously reported by Fehr *et al.*[5]

Patient 5 was a 26-month-old girl. She had experienced partial seizures associated with tonic convulsions at the age of 2 weeks. Her seizures were intractable, and she was diagnosed as suffering from West syndrome at the age of 15 months. A brain MRI at 19 months showed atrophic changes; however, these may have occurred as side effects of adrenocorticotropic hormone therapy (Figures 1c,h,m). This patient was found to have XM_005274584.1: c.1675C>T [p.(Arg559*)] (Figure 2a). This nonsense mutation has been recurrently identified and was reported in our previous study,[7] as well as by Sartori *et al.*[12] and Fehr *et al.*[5]

As described above, we identified pathogenic mutations in *CDKL5* in five unrelated patients with epileptic encephalopathy. All of the parents, except for the father of patient 4, were examined and did not show the mutations identified in the probands. Thus, the *CDKL5* mutations identified in the five patients studied were of *de novo* origin. The detection ratio was 14.8%, which is similar to that reported in our previous study.[7] The gender ratio (four female:one male) of the patients was also similar to that in our previous study.[7] All patients showed intractable epilepsy and moderate to severe developmental delay. Thus, there was no phenotypic difference among the patients with different mutations. Brain MRIs showed no definitive structural abnormality in any of the patients, although brain atrophy was observed in patient 4 at 20 years of age (Figure 1). Although the mechanism of this brain atrophy is unknown, this finding is commonly observed in patients with the *CDKL5* disorder.[7,13]

In this study, all mutations were located at genomic positions where previously reported mutations have been identified. The genomic position of c.404-1G>A, which was first identified in this study, was the same as a previously reported mutation (c.404-1G>T), but the nucleotide alteration pattern of this mutation was different and novel. We confirmed in this study that this splicing acceptor site mutation did cause exon skipping.

In the literature, there are 31 locations in which mutations have been recurrently observed (Figure 2d).[4,14,15] Among them, eight mutations (26%) were at splicing regions distributed throughout the *CDKL5* gene. In contrast, the majority of the recurrent missense mutations are located in the catalytic domains.[6,15] Furthermore, eight missense mutations (26%) were related to C to T (G to A) transitions observed in CG dinucleotide sequences. CG sites are prone to DNA methylation and nucleotide changes.[16] Therefore, these locations are considered as mutation hot spots and would be important for CDKL5 function.

HGV DATABASE

The relevant data from this Data Report are hosted at the Human Genome Variation Database at http://dx.doi.org/10.6084/m9.figshare.hgv.711, http://dx.doi.org/10.6084/m9.figshare.hgv.714, http://dx.doi.org/10.6084/m9.figshare.hgv.717, http://dx.doi.org/10.6084/m9.figshare.hgv.720, http://dx.doi.org/10.6084/m9.figshare.hgv.723.

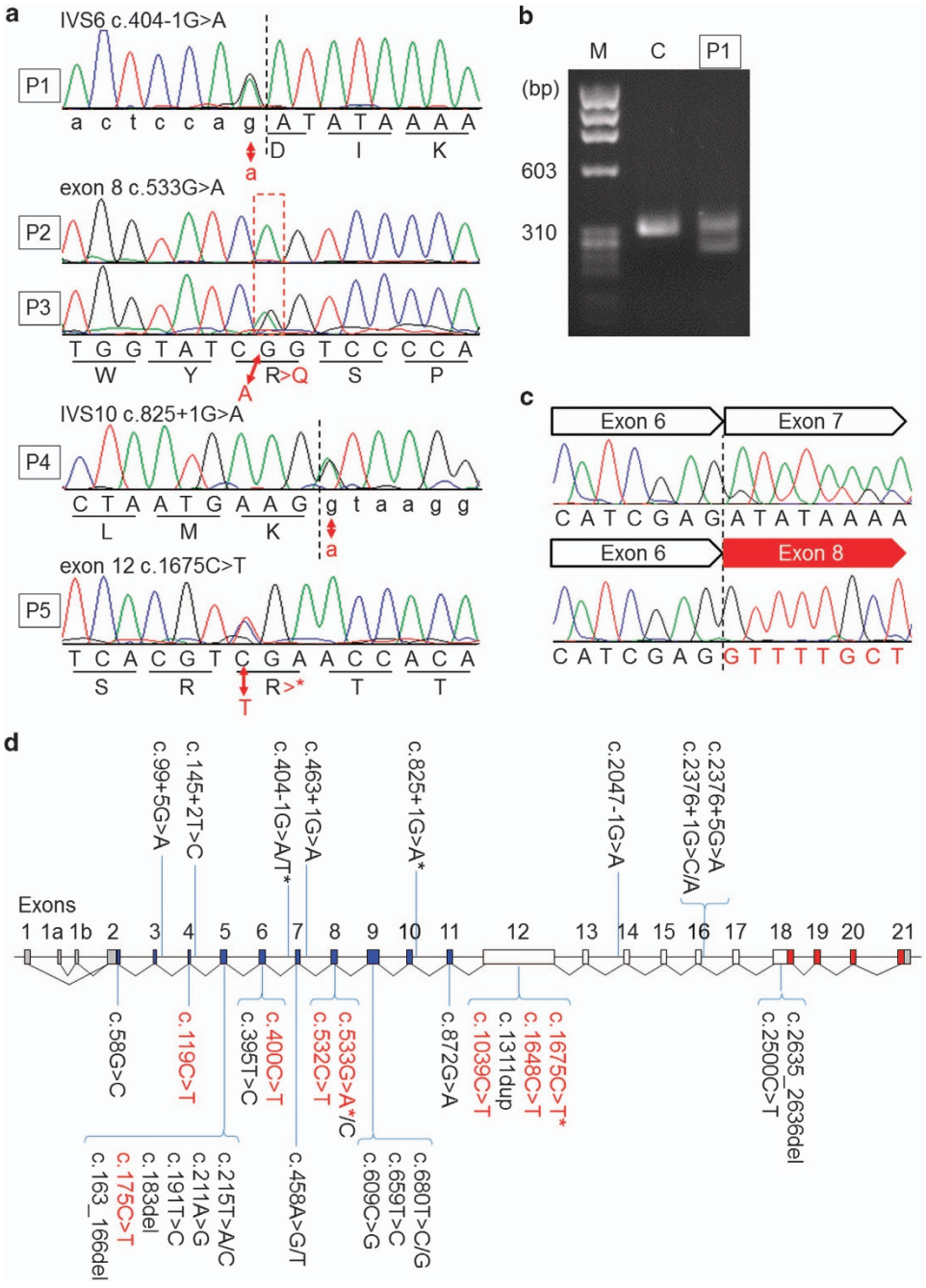

Figure 2. The results of molecular analysis and the locations of the identified mutations. (**a**) Electropherograms of Sanger sequencing for all patients. As patient 2 (P2) was male, he had a hemizygous mutation in c.533G > A (emphasized by a red-dotted box). The remaining patients had heterozygous mutations, as they were female. P1–P5 indicates patient 1–5. Altered nucleotides and amino acids are designated by red characters. Black vertical-dotted lines indicate exon–intron boundaries. (**b**) Agarose gel electrophoresis of the reverse-transcribed (RT)-PCR products. An abnormal short band was observed in the sample from patient 1 (P1). M (marker), phiX174 *Hae*III digest; C, control. (**c**) Sanger sequencing of the two bands obtained from the RT-PCR products of patient 1. Compared with the normal band (upper), the short band showed an abnormal skipping of exon 7 (bottom). (**d**) The recurrently identified mutations are depicted on the primary structure of the *CDKL5* gene. The mutations in the intronic regions and the exonic regions are in the upper and lower portions, respectively. The mutations in red characters are located in the CG dinucleotide sites. Asterisks indicate the mutations identified in this study. Exons are shown by rectangles. Gray, blue and red rectangles indicate nontranslated regions, catalytic domains and C-terminal regions, respectively.

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COMPETING INTERESTS

The authors declare no conflict of interest.

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