CLINICAL REPORTS

Patient 1. The patient was a 4-year-old girl and the first child of healthy, non-consanguineous Japanese parents. The family history was unremarkable. She was born at 40 weeks of gestation weighing 2,564 g and measuring 47.3 cm in length with an occipitofrontal circumference (OFC) of 33 cm, all within the standard range (10th–90th centile) for female Japanese neonates. The child was first evaluated at a cardiology clinic to investigate a heart murmur in the neonatal period. She was diagnosed with Ebstein anomaly, which was surgically repaired when she was 2-month old. At the age of 4 months, she was referred to our hospital due to generalized hypotonia and developmental delay. She rolled over at 12 months and sat up at 18 months. She stood with support at 24 months and started to walk unaided at 2.5 years. At 3 years of age, her height was 84 cm (−2.2 SD), body weight was 12.4 kg (−0.7 SD), and OFC was 49 cm (−0.2 SD). She could speak several meaningful words and understand simple sentences. Her developmental quotient (DQ) was 67, indicating mild intellectual disability. She was a sociable and friendly girl.

Clinical examination revealed that she had a characteristic facial appearance, including a round face, hypertelorism, almond-shaped palpebral fissures, telecanthus, depressed nasal bridge, short nose, microstomia, microretrognathia, short philtrum, and Cupid's bow upper lip (Fig. 1A). Her fingers were slender but not tapered (Fig. 1C). Neurological examination revealed that the cranial nerves were intact except for strabismus. Ocular fundi were normal. She walked slowly, but no ataxia was evident. Muscle tonus of the extremities was normal. Tendon reflexes of extremities were normal, and pathological reflex was absent. There was no evidence of epilepsy. Routine laboratory investigations were normal.

Patient 2. The patient was a 3-year-old girl and was the second child of the parents of Patient 1. She was born at 40 weeks of gestation weighing 2,874 g, measuring 49 cm in length with an OFC of 34.3 cm (all normal values for female Japanese neonates). She exhibited generalized hypotonia, but no feeding problems were observed during the neonatal period. She was referred to our hospital at the age of 19 months due to developmental delay. She exhibited head control at the age of 4 months. She rolled over at 9 months, sat at 10 months, and cruised between 11 and 12 months. She started to walk unaided at 18 months. Her height at 3 years was 88 cm (−2.4 SD), body weight was 10.1 kg (−2.7 SD), and OFC was 47 cm (−0.7 SD). DQ at the age of 3 was 72, indicating mild intellectual disability. She routinely exhibited affectionate and sociable behavior. She also had a round face with full cheeks, hypertelorism, almond-shaped palpebral fissures, telecanthus, depressed nasal bridge, short nose, microstomia, microretrognathia, short philtrum, and Cupid's bow upper lip (Fig. 1B). Ultrasonography of the abdomen showed no urogenital defects. No ophthalmic anomalies other than strabismus were found on routine evaluation. Neurological examination was not remarkable except strabismus. No epileptic seizures were observed. Routine laboratory investigations were normal. The clinical features of both patients and two previously reported cases of 9q34 duplication are summarized in Table I.

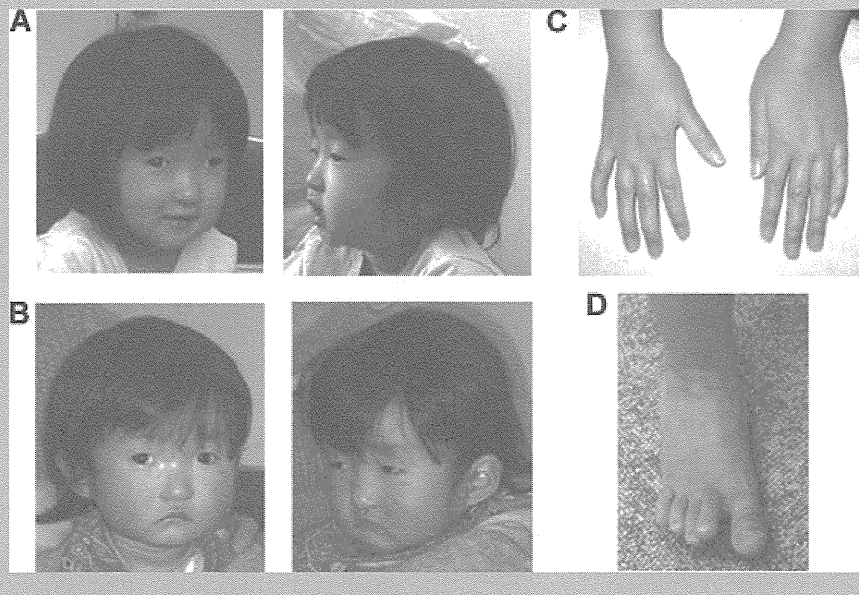

FIG. 1. A: Frontal and lateral views of Patient 1 at 3 years of age. Phenotypes include round face, hypertelorism, telecanthus, short nose, depressed nasal bridge, microstomia, microretrognathia, short philtrum, and Cupid's bow upper lip. B: Frontal and oblique view of Patient 2 at 2 years of age. Phenotypes include round face, hypertelorism, almond-shaped palpebral fissures with telecanthus, short nose, depressed nasal bridge, microstomia, microretrognathia, short philtrum, and Cupid's bow upper lip. C: Hands of Patient 1 with long and thin fingers. D: The right foot of Patient 1. She has long toes with increased space between the first and second toes.

TABLE I. Clinical Features of Patients With a 9q34.1-qter Duplication

Phenotypic features	Gawlik-Kuklinska et al. [2007]	Youngs et al. [2010]	Patient 1	Patient 2
General				
Hypotonia	+	+	+	+
Failure to thrive	+	−	−	−
Intellectual disability	Mild	Mild	Mild	Mild
Cardiac anomalies	−	+	+	−
Overweight/obesity	+	+	−	−
Scoliosis	+	−	−	−
Facial characteristics				
Dolichcephaly	+	+	−	−
Facial asymmetry	+	+	−	−
Narrow horizontal palpebral fissures	+	+	−	−
Deep-set eyes	+	+	−	−
Long nose	+	+	−	−
Prominent chin	+	+	−	−
Microstomia	+	+	+	+
Microretrognathia	+	+	+	+
Short philtrum	+	−	+	+
Round face	−	−	+	+
Hypertelorism	−	−	+	+
Depressed nasal bridge	−	−	+	+
Almond-shape palpebral fissures	−	−	+	+
Telecanthus	−	−	+	+
Short nose	−	−	+	+
Extremities				
Long and thin fingers	+	+	+	+
Increased space between first and second toes	+	+	+	+

+, present; −, absent.

MATERIALS AND METHODS

Cytogenetic Analysis

Cultured lymphoblastoid cells isolated from each patient were treated with colchicine (Sigma–Aldrich, St. Louis, MO) for 1 hr at a concentration of 20 ng/ml in culture medium, and then incubated in a hypotonic solution of 75 mM KCl at 37°C for 30 min. After incubation, cells were fixed with Carnoy's fixative (3:1 mixture of methanol and acetic acid), spread on glass slides in a humid atmosphere and air-dried. Chromosomal analysis was carried out on GTG banded chromosomes at a resolution of 400–550 bands. Fluorescence in situ hybridization (FISH) was performed on metaphase chromosome spreads from each patient. Commercial probes covering subtelomeric regions were used according to the manufacturer's protocols (ToTelVysion, Abbott Laboratories. Abbott Park, IL) [Flint et al., 1995]. In order to confirm the chromosomal rearrangement in detail, additional FISH analysis was carried out from the patients and their parents using a series of bacterial artificial chromosome (BAC) clones (Clontech Laboratories, Inc., Mountain View, CA) that map to chromosome regions 9q34 and 13q31.

Array CGH Analysis

Genomic DNA was isolated from peripheral blood lymphocytes of the two patients, their parents, and three normal controls by phenol/chloroform extraction. Array CGH analysis was performed using the Agilent Human Genome CGH 244K microarray platform (Agilent Technologies, Santa Clara, CA) according to standard protocols provided by the manufacturer. This array spans the entire human genome at a median resolution of approximately 8.9 kb. Genomic copy numbers were analyzed with Genomic Workbench (Standard Edition 5.0.14; Agilent Technologies).

Southern Blot Analysis

Genomic DNA samples (10 μg) from the patients, their parents, and the normal controls were digested with *Hin*dIII, separated on a 0.9% agarose gel, and transferred by the alkaline method to a nylon membrane (Hybond-N+; GE Healthcare, Tokyo, Japan). The membrane was sequentially hybridized with [α-^{32}P]dCTP-labeled *ABCA6* (exons 17–19) and *SP2* (exons 4–7) cDNA. A 301 bp *ABCA6* or a 798 bp *SP2* cDNA probe was prepared by amplifying the cDNA library of human lymphoblastoid cells with AmpliTaq-

Gold (Applied Biosystems, Foster City, CA) using specific primer pairs for *ABCA6* (sense: 5′-ATCTTTTCAGTGATCTGGATAAG-3′; antisense: 5′-AGGGTCAATAACACTTTAGTTT-3′), and for *SP2* (sense: 5′-GTCTACATCCGCACGCCTTC-3′; antisense: 5′-CCGCCGCAGTTGGCCTTA-3′), respectively. The PCR products were subcloned into pGEM-T easy vector (Promega, Madison, WI), and the nucleotide sequence of the probes was confirmed. Hybridization was performed in hybridization solution containing 5× standard saline citrate (SSC), 5× Denhardt's solution, and 0.5% SDS at 66°C overnight. The membrane was washed three times with 2× SSC containing 0.1% SDS at 37°C for 20 min and once with 0.1× SSC containing 0.1% SDS at 55°C for 10 min, and then radioactivity was quantified with a BAS 1800 image analyzer (FUJIFILM, Tokyo, Japan). The radioactivity of *ABCA6* versus *SP2* was determined for both patients and their parents (RP1, RP2, RF, RM) relative to the mean of the three normal controls (RC).

RESULTS

Additional 9q Subtelomeric Signal

The G-banding pattern of the both patients showed a 46,XX normal female karyotype. FISH with probes for subtelomeric regions revealed an additional 9q subtelomeric signal on the short arm of a D-group chromosome (chromosome 13, 14, or 15) in both patients (data not shown).

9q34 Duplication

To assess the chromosomal rearrangements in more detail, FISH analysis was performed in both patients and their parents with three BAC clones (RP11-40A7 and RP11-81N19) from chromosome 9q34 and RP11-524C15 from chromosome 13q31. The result indicated that the mother had a translocation; a 9q34.1-qter segment from one chromosome 9 was translocated to the terminus of chromosome 13p (Fig. 2, lower panel, indicated by a yellow arrow). Both patients had two normal chromosomes 9 and the derivative chromosome 13, which had an additional 9q34.1-qter segment at the p-terminal (Fig. 2, lower panels, indicated by yellow arrows). The father did not show any abnormalities (data not shown). These results indicate that the additional 9q34.1-qter segment at the p-terminal of chromosome 13 was of maternal origin (Fig. 2). The breakpoint of the translocation fell between two BAC clones at RP11-81N19 (129.2 Mb from the 9p terminus) and RP11-40A7 (133.4 Mb). Detailed mapping of the 13p breakpoint is not necessary because 13p does not code any genes. Thus, the duplicated segment was estimated to be 6.8–11.0 Mb derived from the 9q-terminus at position 140.2 Mb [46,XX.ish der(13)t(9;13)-(q34.1;pter)mat] (Fig. 2).

8.5 Mb Duplication of 9q34.1-qter

We performed array CGH using genomic DNA from each patient to determine the precise size of the additional 9q34 segment and

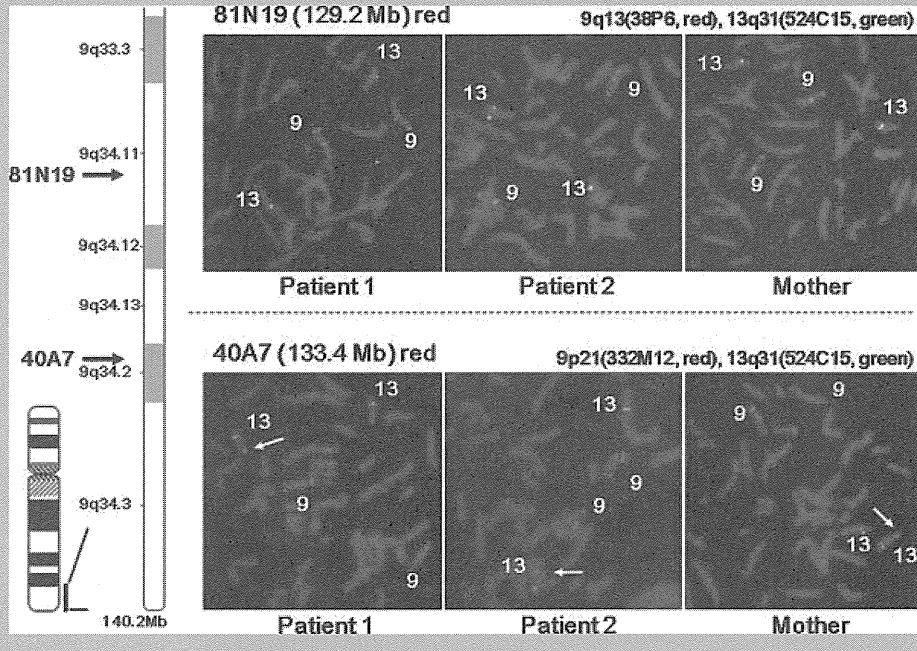

FIG. 2. Partial metaphases of FISH with BAC clone RP11-81N19 probe show two red signals on both 9q terminal regions of the mother and each patient (upper panel) and no signal on chromosome 13. Partial metaphases of FISH with BAC clone RP11-40A7 probe show a red signal on one 9q terminal region and the short arm of derivative chromosome 13 (yellow arrow) in the mother and three signals in both patients; two red signals on both 9q terminal regions and an additional signal on the short arm of derivative chromosome 13 (yellow arrow) (lower panel). RP11-38P6 (red), RP11-332M12 (red), and RP11-524C15 (green) are used as markers for 9q13, 9p21, and 13q31, respectively.

identify any other genomic abnormalities. Array CGH analysis of samples from Patients 1 and 2 demonstrated that the genomic copy number of 9q34.1-qter was 1.5-fold higher than the normal region (Fig. 3A,B). The size of the 9q34.1-qter duplication in both patients was approximately 8.5 Mb, from positions 131.7 to 140.2 Mb of chromosome 9 (Fig. 3). The breakpoint (position 131.7 Mb) of the 9q34 duplication in both patients was located in *FNBP1*, which encodes formin-binding protein 1. Analyses of Patients 1 and 2 revealed 12 and 15 copy number variations (CNVs), respectively (data not shown). CNVs are generally defined as the copy number differences of genomic DNA larger than 1 kb that vary in copy number between individuals. Patients 1 and 2 both had a 0.5-fold decrease in the genomic copy number of *ABCA6*, which encodes ATP-binding cassette, sub-family A, member 6; this is not recognized as a CNV (MIM 612504; Supplemental Fig. A and B).

ABCA6 Deletion in Both Patients and Their Mother

To confirm whether *ABCA6* was deleted in both patients and their parents, we performed Southern blot analysis using two cDNA probes against *ABCA6* (exons 17–19) and *SP2* (exons 4–7). *SP2* maps to 17q21, approximately 21 Mb proximal to *ABCA6*, and was not deleted in either patient based on the array CGH analysis. Southern blot analysis showed a decreased radioactive signal from *ABCA6* in family members (Supplemental Fig. C). When the mean ratio of *ABCA6* signal to *SP2* signal of the three normal controls was defined as 1.0, the ratio of *ABCA6* signal to *SP2* signal of the patients and their mother was approximately 0.5 and their father was 0.85 (Supplemental Fig. D). Thus, the both patients and their mother were heterozygous for an *ABCA6* deletion.

DISCUSSION

Duplications of 9q34 cause intellectual disability and multiple congenital anomalies. Reported cases presented with a variety of clinical features depending on the size of the duplication and the presence of other chromosomal abnormalities [Allderdice et al., 1983; Hodou et al., 1987; Spinner et al., 1993; Gawlik-Kuklinska et al., 2007; Youngs et al., 2010]. Our patients had a 9q34.1-qter duplication and partial 13p monosomy due to a translocation between 9q34.1 and 13pter in their healthy mother. Array CGH and Southern blot analyses confirmed that these patients had a 9q34.1-qter duplication and a heterozygous deletion of *ABCA6* (17q24). Because 13p does not code for any genes and the heterozygous deletion of *ABCA6* did not cause any phenotypic abnormalities in the mother, the present patients exhibited "pure" 9q34.1-qter duplications without any other chromosomal abnormalities involving coding genes.

9q34 duplication has been analyzed in detail using array CGH in only two other patients. Gawlik-Kuklinska et al. [2007] reported the case of the female with a 7.4 Mb (RP11-269P11 to RP11-295G24; 127.3–134.7 Mb) duplication of 9q34.1-q34.3 (Fig. 4) and compared the patient's clinical features to those of previously reported 9q34 duplication cases [Spinner et al., 1993], including a male patient later shown to have a 13.8 Mb (126.4–140.2 Mb) duplication of 9q33.3-qter [Youngs et al., 2010] (Fig. 4). The following

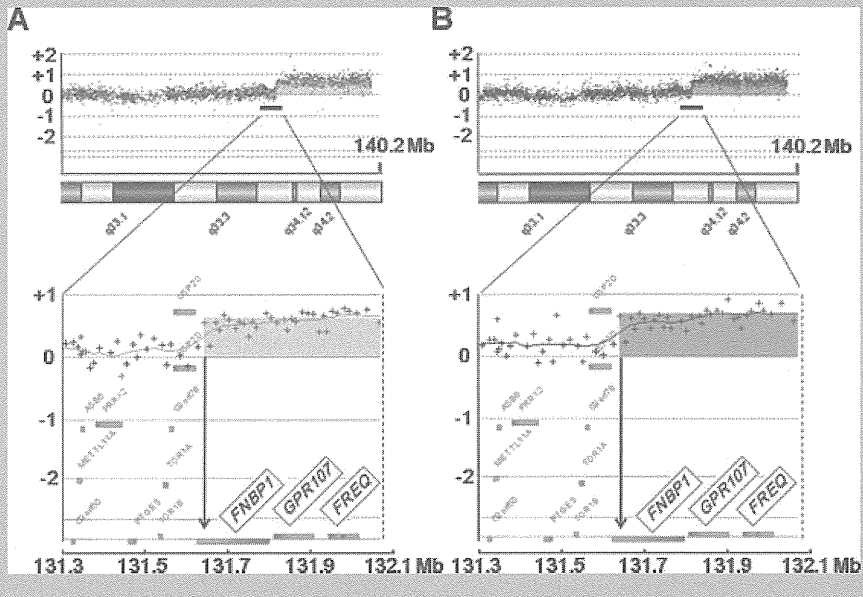

FIG. 3. A: Graphical representation of the results of the array CGH analysis (Agilent 244K oligonucleotide array) from Patient 1 shows the duplication of distal 9q34.1-qter (upper panel). The x- and y-axis denote genomic position and $\log_2$ ratio, respectively. B: Graphical representation of the results of the array CGH analysis from Patient 2 also shows the duplication of distal 9q34.1-qter (upper panel). The breakpoint in 9q34 was located in the *FNBP1* gene (131.7 Mb) in both patients (lower panels of A and B), which indicated that the size of the duplication was approximately 8.5 Mb (131.7–140.2 Mb) according to NCBI human genome build 36.3.

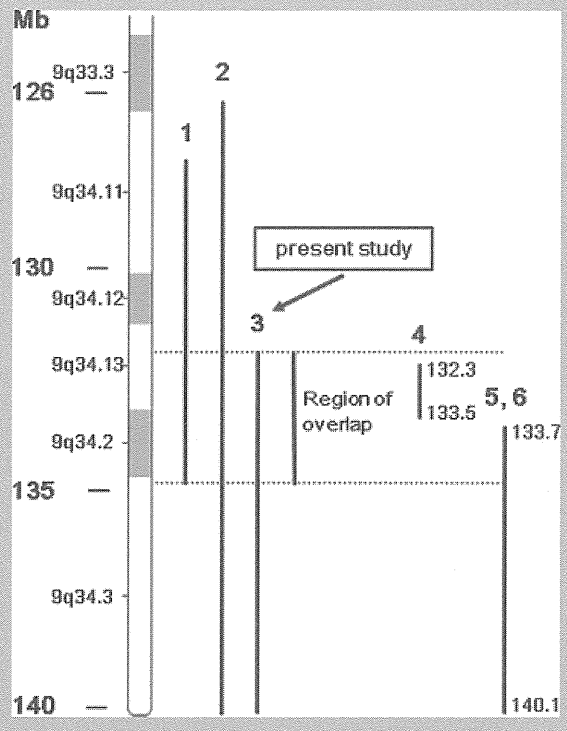

FIG. 4. A schematic illustration based on NCBI human genome build 36.3 of 9q34 duplications from two previously reported cases, the present patients, and three patients from DECIPHER. The duplications in the previously reported patients and our patients are denoted as 1 (127.3–134.7 Mb) [Gawlik-Kuklinska et al., 2007], 2 (126.4–140.2 Mb) [Youngs et al., 2010], and 3 (131.7–140.2 Mb) [present study]. The 3.0 Mb (131.7–134.7 Mb) overlapping region of all three 9q34 duplications is denoted as "Region of overlap." The duplications in the patients from DECIPHER are denoted as 4 (P253579; age 17, 46,XX) (132.3–133.5 Mb), 5 (P254131; age 2, 46,XX) (133.7–140.1 Mb), and 6 (P255167; age 2, 46,XY) (133.7–140.1 Mb).

features were common to both patients in these reports: hypotonia, intellectual disability, developmental delay, characteristic head and facial features associated with dolichocephaly, facial asymmetry, narrow palpebral fissures, deep-set eyes, long nose, prominent chin, microstomia, microretrognathia, and characteristic features of the extremities, including long thin fingers and toes and camptodactyly (Table I). Gawlik-Kuklinska et al. [2007] concluded a 7.4 Mb (127.3–134.7 Mb) duplicated region in their patient was critical for the phenotypes they observed (Fig. 4). Like these two previously reported cases, our patients also exhibited hypotonia, mild intellectual disability, developmental delay, microstomia, microretrognathia, and long thin fingers and toes. Thus, the 3.0 Mb region (131.7–134.7 Mb) of 9q34.13-q34.3 that overlapped in the cases reported by previous studies [Gawlik-Kuklinska et al., 2007; Youngs et al., 2010], and in our patients is most likely associated with the manifestation of the phenotypes observed in all four patients (Fig. 4, Table I). Unlike the other patients, our patients did not have dolichocephaly, facial asymmetry, narrow palpebral fissures, deep-set eyes, or long nose. The locus or loci associated with these phenotypes may be located in a region (127.3–131.7 Mb) that is proximal to the overlapping region (Fig. 4, Table I). Our patients exhibited other characteristic facial features, such as round faces, hypertelorism, almond-shaped palpebral fissures, telecanthus, and short nose; those were not observed in the previously reported cases (Table I). The distal-most segment of 9q34 (134.7–140.2 Mb) in our patients is the strongest candidate for the origin of these phenotypes (Fig. 4). However, these phenotypes were not observed in Patient 2 [Youngs et al., 2010], who had the same 9qter duplication. Therefore, the duplication of the proximal segment (127.3–131.7 Mb) of the overlapping region may have more impact on facial appearance than the duplication of the distal segment of the overlapping region. Clinical analyses of more patients with 9qter duplication (134.7–140.2 Mb) are necessary to determine the phenotypes caused by duplication of this region. It should be noted that DECIPHER (Database of Chromosomal Imbalance and Phenotype in Human using Ensembl Resources) includes two patients (P254131 and P255167) with the same 9q34.2-qter duplication (133.7–140.1 Mb) and heterozygous deletion of 17pter (0.01–0.41 Mb) (Fig. 4, numbers 5, 6). These patients exhibited hypotonia (non-myopathic), intellectual disability, developmental delay, patchy café au lait pigmentation spots on the skin, and speech delay. The heterozygous 17pter 0.4 Mb deletion has not been reported to cause any diseases, including intellectual disability. Another patient (P253579) presenting with facial abnormality, intellectual disability, and developmental delay had a 9q34.1-q34.2 duplication (132.3–133.5 Mb) in the 3.0 Mb overlapping region (Fig. 4, number 4). Notably, these two duplicated regions are included in the duplicated region in our patients, but they do not overlap with each other. These findings suggest the following correlations between duplicated chromosomal segments of 9q34 and phenotypes: (1) two duplicated segments (133.7–140.1 and 132.3–133.5 Mb) in 9q34 are associated with intellectual disability and developmental delay; and (2) the locus or loci associated with characteristic facial appearance may be within a duplicated region of 1.2 Mb (132.3–133.5 Mb), even though the detailed clinical features of P253579 are not available. Of the 18 genes that map to this 1.2 Mb region, individual duplications of 12 genes are reported in the Database of Genomic Variants (DGV; found in normal population). Thus, increased copy number of one or more of the other six genes (*FUBP3*, *EXOSC2*, *ABL1*, *NUP214*, *FAM78A*, and *PPAPDC3*) in this region could be the cause of the intellectual disability, developmental delay, and characteristic facial appearance observed in our patients and P253579.

Chromosomal rearrangements, arising from unequal recombination between repeated sequences, are found in a subset of patients with autism spectrum disorder [Marshall et al., 2008]. Abnormal behaviors, including hyperactive behavior [Allderdice et al., 1983], food-seeking behavior [Gawlik-Kuklinska et al., 2007], hyperactivity, attention deficit disorders, and atypical autism [Youngs et al., 2010], were also reported in some patients with 9q34 duplication. Unlike these patients, our patients exhibited friendly and affectionate social behaviors and did not exhibit autistic features or attention deficit disorder. It is important to repeatedly monitor the behaviors

of our patients to determine whether the 9q34.1-qter duplication is associated with abnormal behaviors. In summary, our findings indicate that the duplication of 9q34 is a heterogeneous clinical condition and duplications of different segments of 9q34 are associated with a variety of symptoms. Genomic and clinical analyses of more patients carrying 9q34 duplications are necessary to better characterize the correlation between clinical phenotypes and specific 9q34 loci.

ACKNOWLEDGMENTS

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FULL-LENGTH ORIGINAL RESEARCH

CDKL5 alterations lead to early epileptic encephalopathy in both genders

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Summary

Purpose: Genetic mutations of the cyclin-dependent kinase-like 5 gene (*CDKL5*) have been reported in patients with epileptic encephalopathy, which is characterized by intractable seizures and severe-to-profound developmental delay. We investigated the clinical relevance of *CDKL5* alterations in both genders.

Methods: A total of 125 patients with epileptic encephalopathy were examined for genomic copy number aberrations, and 119 patients with no such aberrations were further examined for *CDKL5* mutations. Five patients with Rett syndrome, who did not show methyl CpG-binding protein 2 gene (*MECP2*) mutations, were also examined for *CDKL5* mutations.

Key Findings: One male and three female patients showed submicroscopic deletions including *CDKL5*, and two male and six female patients showed *CDKL5* nucleotide alterations. Development of early onset seizure was a characteristic clinical feature for the patients with *CDKL5* alterations in both genders despite polymorphous seizure types, including myoclonic seizures, tonic seizures, and spasms. Severe developmental delays and mild frontal lobe atrophies revealed by brain magnetic resonance imaging (MRI) were observed in almost all patients, and there was no gender difference in phenotypic features.

Significance: We observed that 5% of the male patients and 14% of the female patients with epileptic encephalopathy had *CDKL5* alterations. These findings indicate that alterations in *CDKL5* are associated with early epileptic encephalopathy in both female and male patients.

KEY WORDS: *CDKL5*, Epileptic encephalopathy, Genomic copy number aberration, Mutation, Gender.

Epileptic encephalopathies are a group of conditions in which neurologic deterioration results mainly from epileptic activity. The clinical and electroencephalography (EEG) characteristics depend on the age of onset and may change over time (Zupanc, 2009). An underlying genetic background has been suggested in patients with epileptic encephalopathy (Nabbout & Dulac, 2008). An X-linked gene coding for cyclin-dependent kinase-like 5 gene (*CDKL5*; MIM #300203) is one of the genes responsible for epileptic encephalopathy. Kalscheuer et al. (2003) identified de novo balanced X autosome translocations in two female patients with infantile spasms, in whom *CDKL5* was disrupted. Since then, the phenotypic spectrum of *CDLK5* abnormalities has expanded to include features resembling Rett syndrome (RTT; MIM #312750) with early onset seizures (Evans et al., 2005; Mari et al., 2005). Now, phenotypic features of *CDLK5* abnormalities are widely recognized as early infantile epileptic encephalopathy-2 (EIEE-2; MIM #30062) and are characterized as severe epileptic encephalopathy associated with early onset and refractory seizures (Archer et al., 2006; Pintaudi et al., 2008).

Although the consequence of *CDKL5* alterations has also been attributed to X-linked dominant infantile spasm syndrome-2 (ISSX2), mutations have been identified not only in female patients but also in some male patients with severe mental retardation and early onset intractable seizures (Elia et al., 2008; Fichou et al., 2009; Sartori et al., 2009).

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Therefore, we performed a comprehensive analysis for *CDKL5* in both female and male patients with epileptic encephalopathy.

Methods

Patients

After obtaining approval of the study protocol by the ethics committee of the institution and informed consent from the families of the patients, peripheral blood samples of 125 patients (59 male and 66 female) with epileptic encephalopathy of unknown etiology were collected, together with their clinical information, including neuroimaging findings. Epileptic encephalopathies are defined as disorders in which there is a temporal relationship between deterioration in cognitive, sensory, and motor function and epileptic activity, which includes frequent seizures and/or extremely frequent interictal paroxysmal activity (Nabbout & Dulac, 2003). Five female patients with RTT who did not show methyl CpG-binding protein 2 gene (*MECP2*) mutations (which are often associated with RTT) were also included in the cohort study for *CDKL5* mutations.

Microarray-based comparative genomic hybridization (aCGH) analysis

The genomic copy numbers of the patients with epileptic encephalopathies were determined using the Human Genome CGH Microarray 105K (Agilent Technologies, Santa Clara, CA, U.S.A.) as described previously (Shimojima et al., 2010).

Validation of the genomic copy number aberrations

Fluorescent in situ hybridization (FISH) analysis was performed for the large chromosomal deletion by using bacterial artificial chromosome (BAC) clones as probes, RP11-106N3 and CTD-2335C24 including *CDKL5* as a target, and RP11-1051J20 as a marker (Fig. 1, Table S1). The deletion identified in Patient 1 was too small to be detected by a BAC clone; therefore, multiplex polymerase chain reaction (PCR) analysis was used for validation. Two DNA fragments, exon 1B (421 bp) and exon 2 (350 bp) of *CDKL5*, were amplified in the same PCR reaction tube, separated by agarose gel electrophoresis, and visualized by ethidium bromide staining.

Cohort study for *CDKL5*

Samples from 119 patients (58 male and 61 female) that showed no genomic copy number aberrations at the first screening by microarray-based comparative genomic hybridization (aCGH) in this study were included in the second cohort. Five samples obtained from female patients with RTT who did not show *MECP2* mutations were also included. The genomic sequences of all 23 exons of *CDKL5* were analyzed by the standard PCR direct-sequencing method using primers listed in Table S2. A recently identified exon 16B, which if included in the mature mRNA produces as a new *CDKL5* isoform, was also analyzed in this study (Fichou et al., 2010). When nucleotide changes were identified in samples for which parental samples were available, trio analyses were performed to test whether the mutation was de novo or familial. DNA samples collected from 100 healthy Japanese volunteers (50 male and 50 female) comprised the control cohort.

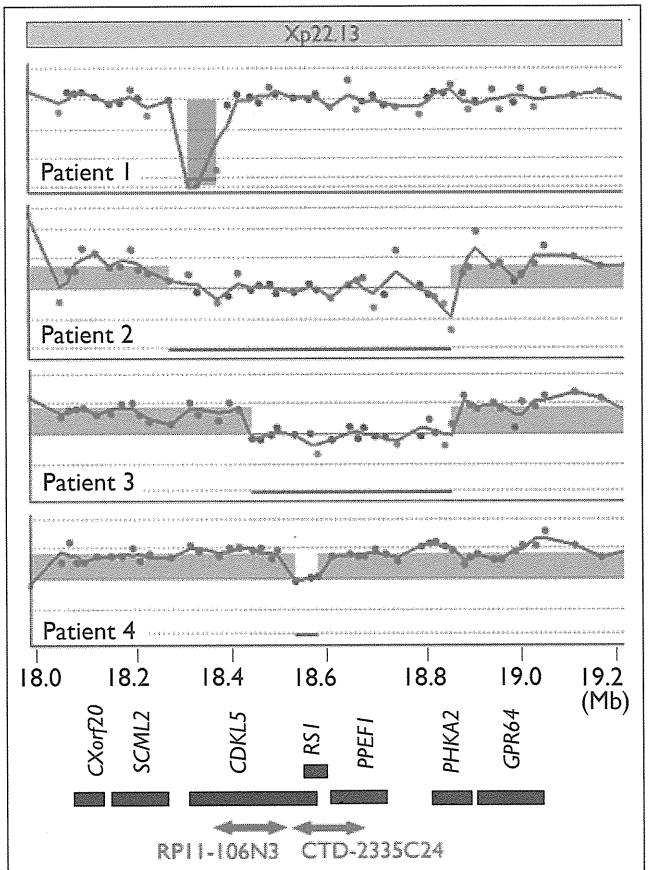

Figure 1.
Results of aCGH. The *CDKL5* region was expanded in Gene View with Agilent Genomic Workbench software (Agilent Technologies). The *x* and *y* axes indicate chromosomal positions and $\log_2$ ratios of intensity, respectively. Moving averages are shown in blue and red lines in male and female patients, respectively. Dots indicate the positions and $\log_2$ ratio of the probes. Rectangles and bars with arrows in both edges indicate the locations of the genes and BAC clones used for FISH, respectively. All patients show genomic copy number aberrations in the *CDKL5* region.

Results

Genomic copy number aberrations

In Patient 1, an aberration was identified at Xp22.13, indicating a nullisomy of this region (Fig. 1, Table S3). This region corresponds to exon 1 of *CDKL5*. Subsequent

multiplex PCR analysis using two sets of primers for exon 1B and exon 2 of *CDKL5* showed no band for exon 1B (Fig. 2A), thereby confirming the nullisomy of this region. Both parents of Patient 1 declined trio analysis.

aCGH analysis identified chromosomal aberrations in the *CDKL5* region in three female patients (Fig. 1, Table S3). Because male reference DNA was used in this study, genomic copy numbers of the normal female X chromosome regions showed $\log_2$ ratio of +1. Therefore, a $\log_2$ ratio of "0" indicates the same genomic copy numbers with the male reference sample, indicating a partial monosomy of this region in these patients. For Patients 2 and 3, identified aberrations were confirmed by FISH by detecting only one signal with RP11-106N3 and CTD-2335C24, respectively, indicating deletions in this region (Fig. 2B,C). For Patient 4, one of the targeted signals of CTD-2335C24 was weaker than the other, indicating a partial deletion of the targeted region (Fig. 2D). For Patients 2 and 3, the deletion region involved four genes: *CDKL5*; X-linked juvenile retinoschisis protein gene (*RS1*), which is responsible for X-linked juvenile retinoschisis (MIM #312700); protein phosphatase with EF hand calcium-binding gene (*PPEF1*); and phosphorylase kinase alpha 2 gene (*PHKA2*), which is responsible for X-linked hepatic glycogen storage disease (MIM #300798). For Patient 3, the deleted region involved the

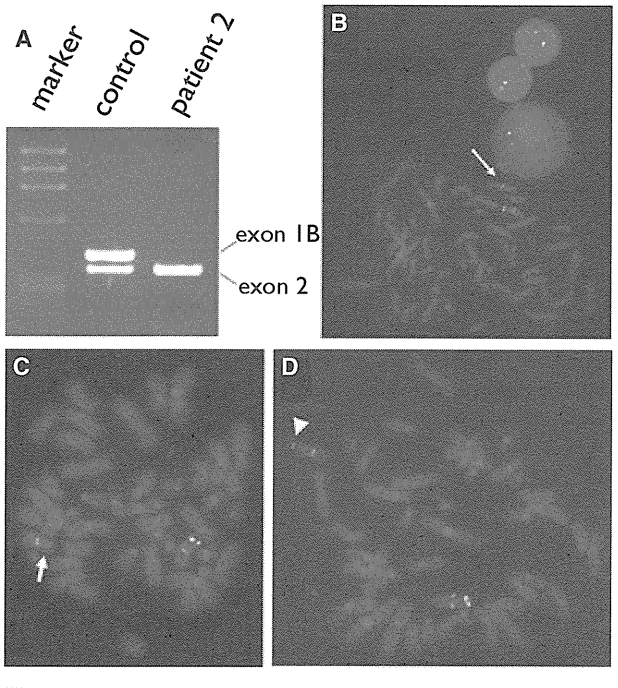

Figure 2.
Validations of genomic copy number aberrations. (**A**) Multiplex PCR amplification indicates deletion of exon 1B in Patient 1. The marker lane shows HaeIII digested φX174 DNA. (**B**, **C**) FISH analysis indicates loss of the green signal on one of the X chromosomes (arrows). For Patient 2 (**B**) and Patient 3 (**C**), RP11-106N3 and CTD-2335C24 are used for the targets, respectively. Patient 4 (**D**) shows a week green signal labeled on CTD-2335C24 (arrowhead), indicating a partial deletion within CTD-2335C24 region.
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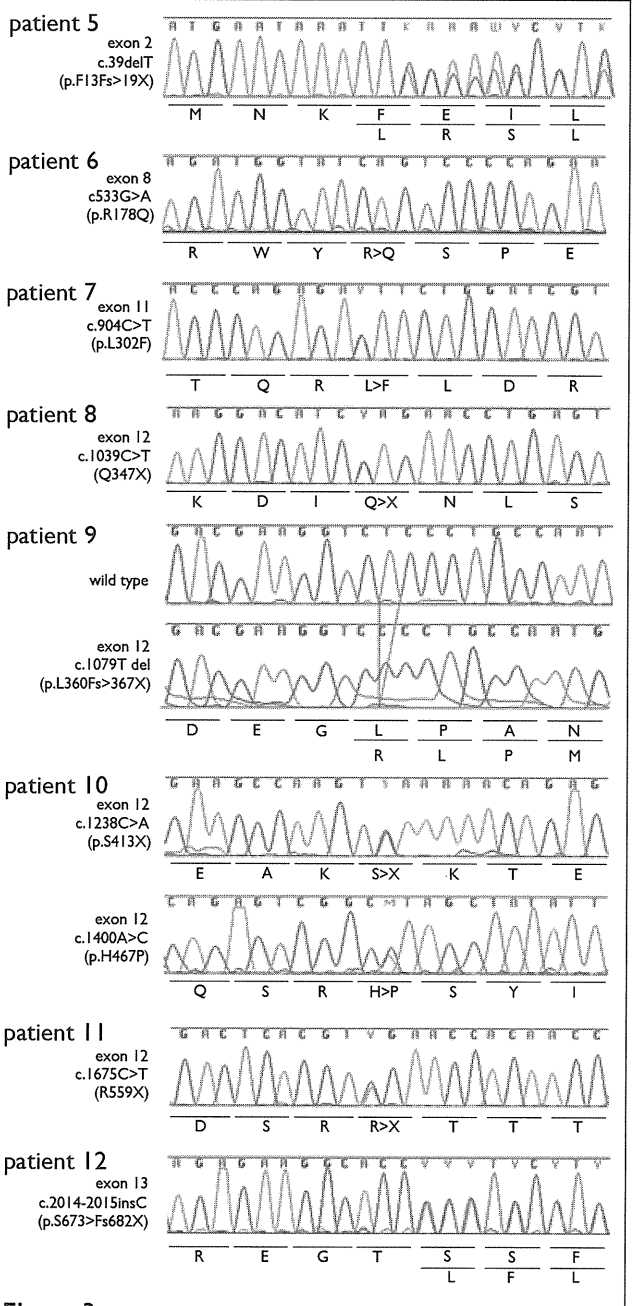

Figure 3.
Electrophoresis of the direct sequencing. Alphabetic symbols indicate amino acids. For Patients 5, 9, and 12, lines above the sequences indicate reference amino acid sequences, and lines below the sequences indicate amino acid changes caused by the mutations.
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Table 1. Summary of the clinical features and the identified *CDKL5* mutations in the patients reported in this study

No.	1	2	3	4	5	6	7	8	9	10	11	12
Gender	M	F	F	F	F	M	F	F	M	F	F	F
Initial concerns	EE	EE	EE	EE	EE	EE	EE	EE	EE	EE	EE	EE
Age at examination	6 m	2 y 7 m	4 y 2 m	2 y 7 m	8 m	1 y 9 m	4 y 7 m	2 y 6 m	2 y	2 y 1 m	1 y 4 m	1 y 4 m
Physical examination												
Birth weight (g)	3,458	3,016	2,400	2,716	2,612	3,800	2,560	3,352	3,228	2,955	3,250	2,976
OFC at birth (cm)	36.0	36.0	32.0	32.0	30.3	34.0	NT	NT	36.0	NT	33.0	33.5
Microcephaly	–	–	+	+	+	–	–	–	–	–	–	+
Deceleration of head growth	–	–	–	–	+	–	–	–	–	–	–	+
Neurologic features												
Hypotonia	–	–	+	+	+	+	+/–	+/–	+	+/–	+	+
Autistic features	NT	NT	+	NT	NT	–	+/–	+	NT	+	+	NT
Stereotype movement	NT	NT	+	+	+	–	–	+	NT	+	–	NT
Development												
Sitting	–	–	–	–	–	–	–	+	–	–	–	+
Walking	–	–	–	–	–	–	–	–	–	–	–	–
Best motor development	Bedridden	Bedridden	Turn over	Turn over	Bedridden	Bedridden	Bedridden	Sit	Bedridden	Turn over	Turn over	Sit
Speech	–	–	–	–	–	–	–	–	–	–	–	–
Seizure												
Age at onset of seizure	1 m	1 m	2 m	1.5 m	2 w	2 w	4 d	2 m	3 m	3 w	6 m	6 w
Persistent epilepsy	+	+	+	+	+	+	–	+	+	+	+	+
Seizure type	Infantile spasms	Infantile spasms	Spasms, focal Sz, myoclonia	Spasms, focal Sz	Spasms, focal Sz	Epileptic spasms	Infantile spasms	Tonic–clonic convulsion	Infantile spasms	Tonic–clonic convulsion	Tonic–clonic convulsion	Epileptic spasms
Radiologic examination												
Brain MRI	Cerebral atrophy	Cerebral atrophy	Cerebral atrophy	Mild cerebral atrophy	Cerebral atrophy	Bifrontal-diffuse atrophy	Very mild cerebral atrophy	Mild frontal lobe atrophy	Cerebral atrophy	Cerebral atrophy	Frontal lobe atrophy and delayed myelination	Mild cerebral atrophy
Hypoperfusion revealed by SPECT	NT	NT	Left frontal	NT	No abnormality	Right frontal	Right temporal	Left frontal	No abnormality	NT	Frontal and left parietal	NT

Continued

Table 1. Continued

No.	1	2	3	4	5	6	7	8	9	10	11	12
Mutation												
Location	Exon 1	Whole exons	Large deletion after exon 4	Large deletion after exon 16	Exon 2	Exon 8	Exon 11	Exon 12	Exon 12	Exon 12	Exon 12	Exon 13
Nucleotide change					c.39delT	c.533G>A	c.904C>T	c.1039C>T	c.1079delT	c.1238C>G	c.1400A>C	c.1675C>T	c.2014-2015insC
Amino acid change					p.F13Fs>19X	p.R178Q	p.L302F	p.Q347X	p.L360Fs>367X	p.S413X	p.H467P	p.R559X	p.S673>Fs682X
Domain						Catalytic							
Inheritance	NT	De novo	De novo	De novo	NT	De novo	NT	NT	NT	De novo	De novo	NT	De novo
Novel/recurrent	Novel	Novel	Novel	Novel	Novel	Novel	Novel	Recurrent	Novel	Novel	Novel	Recurrent	Novel
Population study	NT	NT	NT	NT	None	None	None	None	None	None	None	None	None
Previous reports								Artuso et al. (2010)				Sartori et al. (2009)	

M, male; F, female; EE, epileptic encephalopathy; y, years; m, months; w, weeks; d, days; OFC, occipitofrontal circumference; NT, not tested; Sz, seizures; SPECT, single-photon emission computed tomography.

latter half of *CDKL5* after exon 4. Patient 4 also showed a partial *CDKL5* deletion after exon 16, and *RS1*, which was encoded in the antisense direction. For Patients 2, 3, and 4, both parents were negative for these deletions, indicating de novo origin.

There were no other known pathogenic aberrations in these four patients. In the other two patients, genomic copy number aberrations in the region of the platelet-activating factor acetylhydrolase gene (*PAFAH1B1*), which is responsible for lissencephaly, were identified (Shimojima et al., 2010). The remaining 119 patients showed no genomic copy number aberrations and were included in the cohort study for *CDKL5* mutations.

CDKL5 nucleotide alterations

In the 119 patients, eight pathogenic mutations were identified (including six novel and two recurrent mutations), which consisted of three nonsense mutations, three frameshift mutations, and two missense mutations (Fig. 3, Table 1). Aristaless-related homeobox gene (*ARX*; MIM #300382) was not found in any of the male patients. Five patients with RTT who did not show *MECP2* mutations also did not show mutations in *CDKL5*. No control samples showed any of the nucleotide alterations identified in this study (Table 1).

Although Patient 10 showed a nonsense mutation (p.S413X), an additional missense mutation (p.H467P) was also identified in exon 12. Neither alteration was found in parents, indicating de novo occurrence of both mutations. Because a similar missense mutation (p.H467R) was reported to be a nonpathogenic mutation, p.H467P is also expected to be a nonpathogenic mutation (Evans et al., 2005).

Clinical description

Brain magnetic resonance imaging (MRI) of the patients with *CDKL5* alterations is shown in Fig. 4. Many patients showed frontal dominant cerebral atrophy. All clinical data including the findings of neuroimaging are summarized in Table 1. The ability to sit autonomously was the maximum gross motor development achieved by these patients, and none of the patients acquired speech ability, indicating severe developmental delay. Only the oldest patient (Patient 7; 4 years and 7 months old), who had a missense mutation, showed seizure control after 3 years of age; all the other patients had persistent seizures.

Discussion

Using aCGH analyses, Erez et al. (2009) identified partial *CDKL5* deletions in female patients with early onset intractable epilepsy. Mei et al. (2010) identified four patients who had total or partial deletions in *CDKL5*. However, those studies included only female patients. In comparison, the aim of our study was to identify candidate

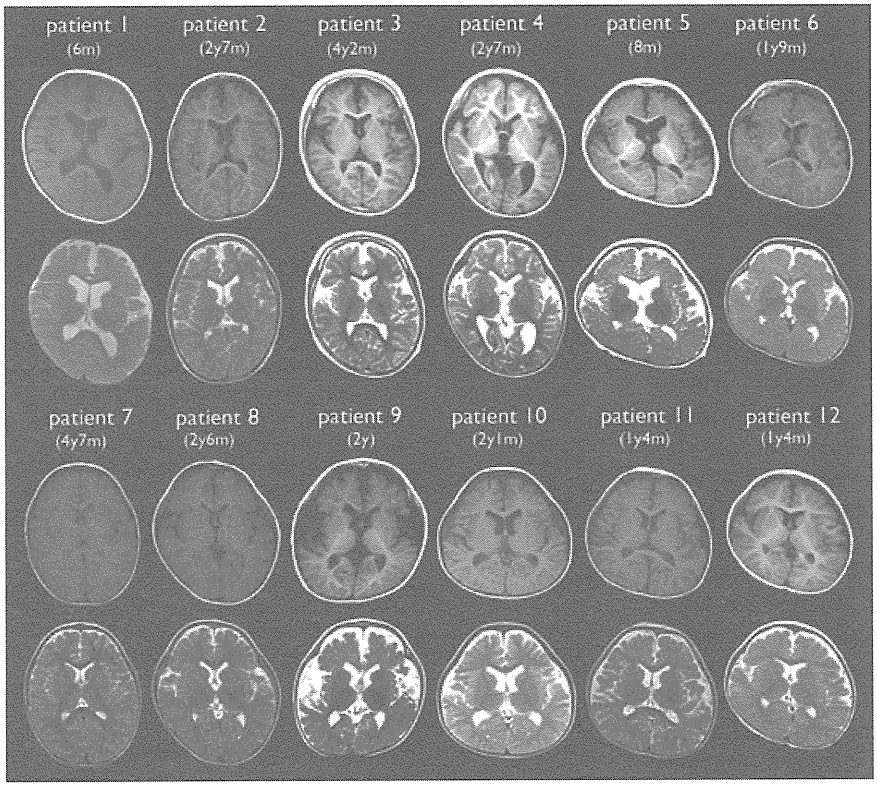

Figure 4.
Brain MRI findings of the patients. T_1- (up) and T_2-weighted (bottom) MRI indicates frontal atrophies in many patients, except for Patient 7. In Patient 6, spoiled gradient echo (SPGR) is shown instead of T_1.
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genetic causes of early epileptic encephalopathy, and thus we recruited patients of both genders. Genomic copy numbers of whole chromosomes were comprehensively analyzed and submicroscopic chromosomal abnormalities of the *CDKL5* region were identified in both genders. The male patient (Patient 1) showed a partial deletion of *CDKL5*. Patients 2 and 3 showed large deletions in which the four neighboring genes, *CDKL5*, *RS1*, *PPEF1*, and *PHKA2*, were included. *RS1* and *PHKA2* are responsible for X-linked diseases, and the function of *PPEF1* is unknown. The remaining Patient 4 showed partial deletions of *CDKL5* and *RS1*. Therefore, phenotypic features of Patients 2, 3, and 4 suggest a causal role for *CDKL5* deletions in early epileptic encephalopathy. Despite the gender difference and the deleted size differences, the clinical severities of the patients with *CDKL5* deletions were similar between genders and similar to those of patients previously reported to have partial or total deletion of *CDKL5* (Van Esch et al., 2007; Erez et al., 2009; Bahi-Buisson et al., 2010; Mei et al., 2010).

Previously, *CDKL5* mutations were shown to affect mainly female patients, and their frequency has been estimated as approximately 9–28% in female patients with early onset seizures (Bahi-Buisson et al., 2008b; Nemos et al., 2009). However, those studies mainly included female patients. Elia et al. (2008) identified *CDKL5* mutations in three male patients with early onset epileptic encephalopathy. Male patients with *CDKL5* mutations or deletions have also been reported by others (Fichou et al., 2009; Sartori et al., 2009). In our study, initial identification of *CDKL5* deletions in both male and female patients with early epileptic encephalopathy prompted us to analyze *CDKL5* nucleotide sequences of both genders, and the results revealed nucleotide changes in two male patients and six female patients. We observed that the clinical severity of the disease did not differ between males and females. Therefore, male as well as female patients with early onset epileptic encephalopathy should be tested for *CDKL5* mutations.

Because *CDKL5* is located on Xp22.13, genetic traits of *CDKL5* alterations have been considered to be X-linked dominant, just as *MECP2* mutations are responsible for the majority of RTT cases, a neurologic disorder occurring almost exclusively in females. The rare male patients with *MECP2* mutations showed severe mental retardation but no RTT phenotype (Gomot et al., 2003). In comparison, there are no phenotypic differences between male and female patients with *CDKL5* mutations or deletions. Bahi-Buisson et al. (2008b) suggested that phenotypic heterogeneity does not correlate with the nature or the position of the mutations or with the pattern of X-chromosome inactivation. Indeed, no clear genotype–phenotype correlation between these factors has been established. Therefore, an important question is why clinical severity is the same between the genders. Based on previous reports, we know that the absence of CDKL5 protein is not lethal in males, and *CDKL5*

abnormalities result in severe neurodevelopmental delay and early onset epilepsy in both genders (Castren et al., 2011). In this study, the estimated frequencies of *CDKL5* abnormalities in patients with epileptic encephalopathy were 5% in male and 14% in female patients. Therefore, the observed difference in the frequency of *CDKL5* mutations between male and female patients may simply be a consequence of the fact that female patients have two X chromosomes.

Subjects in our study included five female patients with RTT who did not show *MECP2* mutations. However, these female patients did not carry a *CDKL5* mutation. Some researchers have found no *CDKL5* mutations in patients with RTT (Huppke et al., 2005; Li et al., 2007). Previously, *CDKL5* mutations were analyzed in patients with both classic and atypical variants of RTT. However, mutations were identified only in patients with seizure onset before 6 months of age (Evans et al., 2005; Scala et al., 2005; Artuso et al., 2010). In another study, all patients with *CDKL5* mutations showed early onset seizures that began before 6 months of age (Erez et al., 2009). These findings suggest that development of early onset seizures is an essential clinical feature in patients with *CDKL5* mutations. The onset of epileptic seizures in the first 6 months distinguishes patients with *CDKL5* mutations from patients with typical RTT caused by *MECP2* mutations (Castren et al., 2011).

All previously reported *CDKL5* mutations were sporadic and were identified as de novo. Only a small numbers of mutations were recurrent (Castren et al., 2011). In this study, we observed eight *CDKL5* mutations that included six novel and two recurrent mutations. The phenotypic features of the patients with recurrent mutations are similar to those described previously (Sartori et al., 2009; Artuso et al., 2010).

Consistent with the findings of previous studies, we observed polymorphous seizures (i.e., myoclonic seizures, tonic seizures, and spasms) in our study. The clinical course of seizure development was also identical to the proposed three stages reported by Bahi-Buisson et al. (2008a) [i.e., stage I, early onset epilepsy (onset 1–10 weeks); stage II, epileptic encephalopathy with infantile spasms and hypsarrhythmia; stage III, seizure-free in estimated 50% of patients at late infantile period] because our Patient 7 showed good seizure control after 3 years of age. Artuso et al. (2010) reported that patients with *CDKL5* mutations showed no abnormalities on brain magnetic resonance imaging (MRI). However, our findings indicated mild frontal lobe atrophy in almost all patients. Therefore, this may be an additional clinical characteristic of patients with *CDKL5* mutations.

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Disclosure

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. The physical positions of BAC clones.

Table S2. Primer sequences for *CDKL5*.

Table S3. The results of aCGH.

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