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

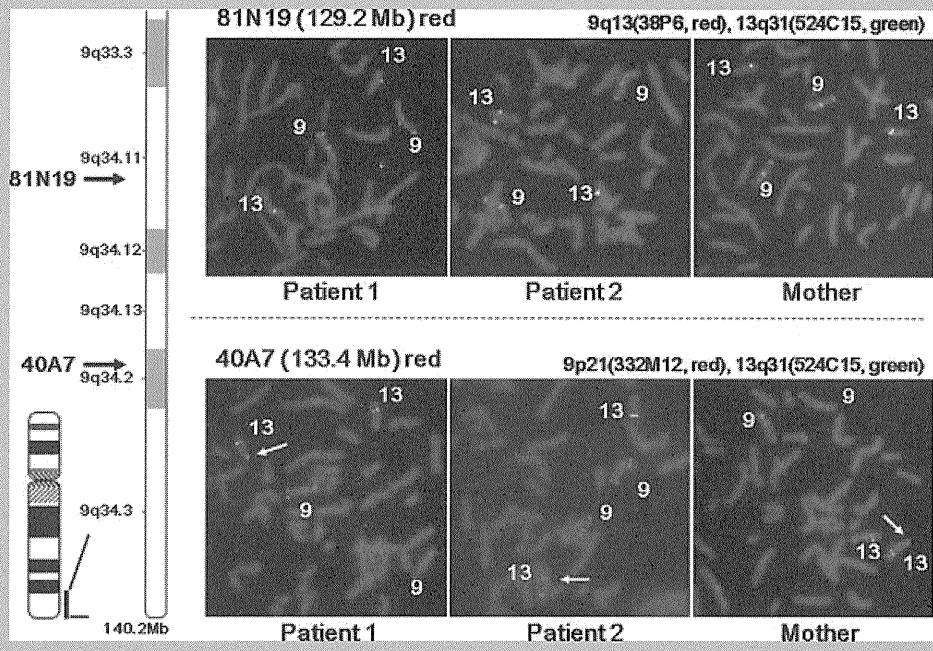

FIG. 2. Partial metaphases of FISH with BAC clone RP11-81N19 probe show two red signals on both 9q terminal regions of the mother and each patient (upper panel) and no signal on chromosome 13. Partial metaphases of FISH with BAC clone RP11-40A7 probe show a red signal on one 9q terminal region and the 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

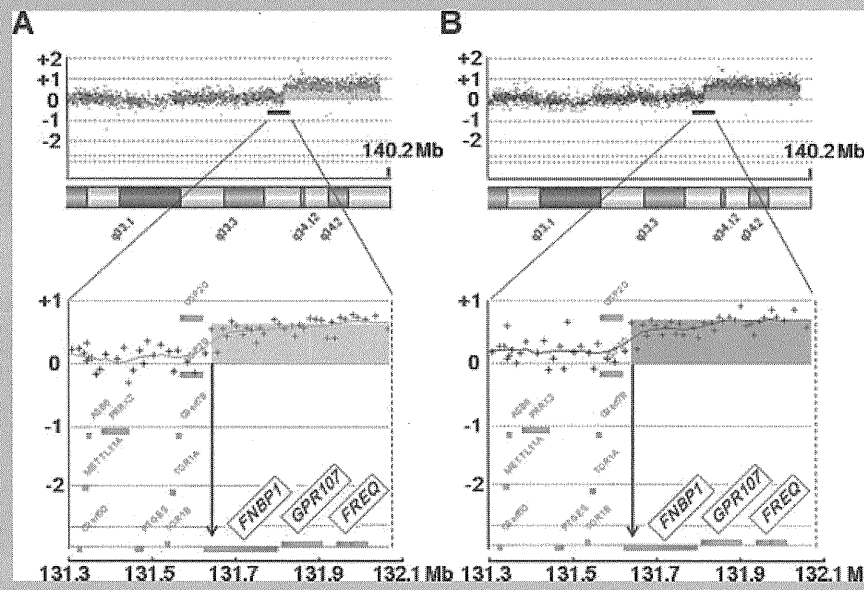

FIG. 3. A: Graphical representation of the results of the array CGH analysis (Agilent 244K oligonucleotide array) from Patient 1 shows the duplication of distal 9q34.1-qter (upper panel). The x- and y-axis denote genomic position and $\log_2$ ratio, respectively. B: Graphical representation of the results 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.

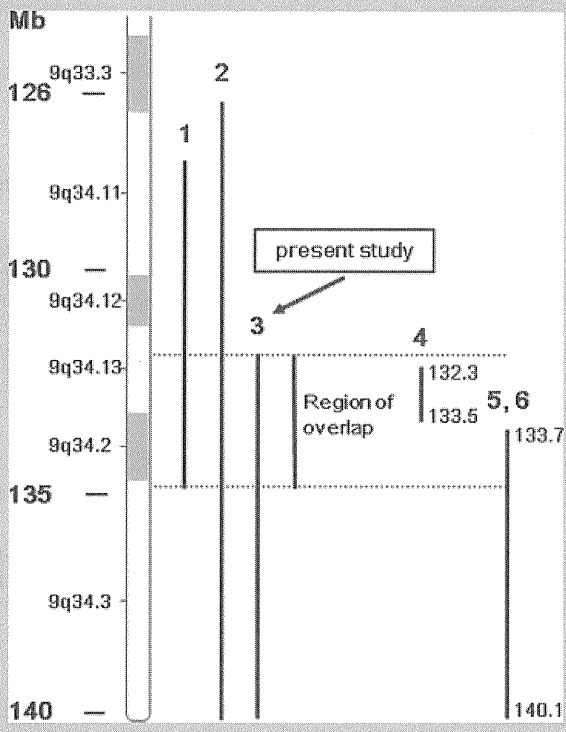

FIG. 4. A schematic illustration based on NCBI human genome build 36.3 of 9q34 duplications from two previously reported cases, the present patients, and three patients from DECIPHER. The duplications in the previously reported patients and our patients are denoted as 1 (127.3–134.7 Mb) [Gawlik-Kuklinska 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.

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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.

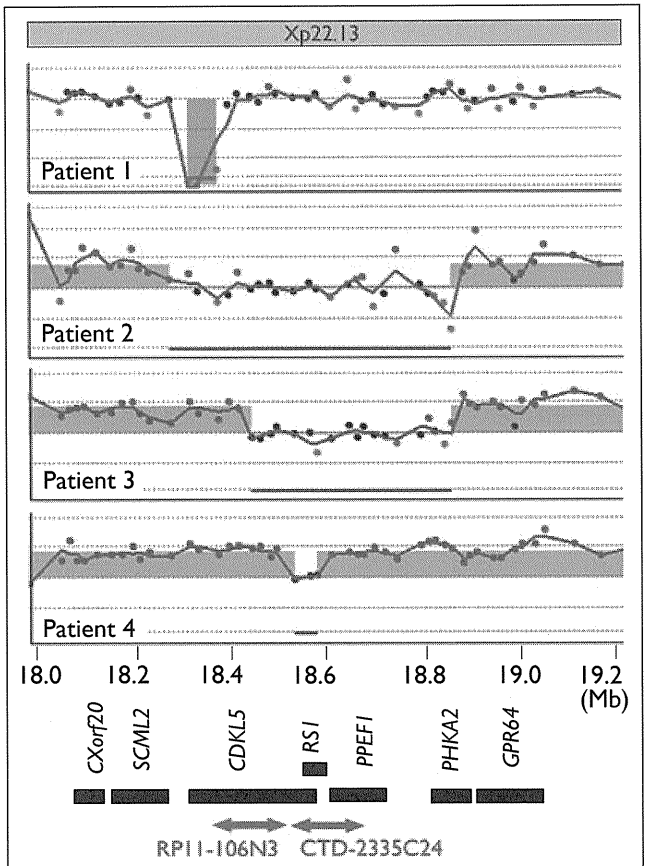

Figure 1.
Results of aCGH. The *CDKL5* region was expanded in Gene View with Agilent Genomic Workbench software (Agilent Technologies). The *x* and *y* axes indicate chromosomal positions and $\log_2$ ratios of intensity, respectively. Moving averages are shown in blue and red lines in male and female 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

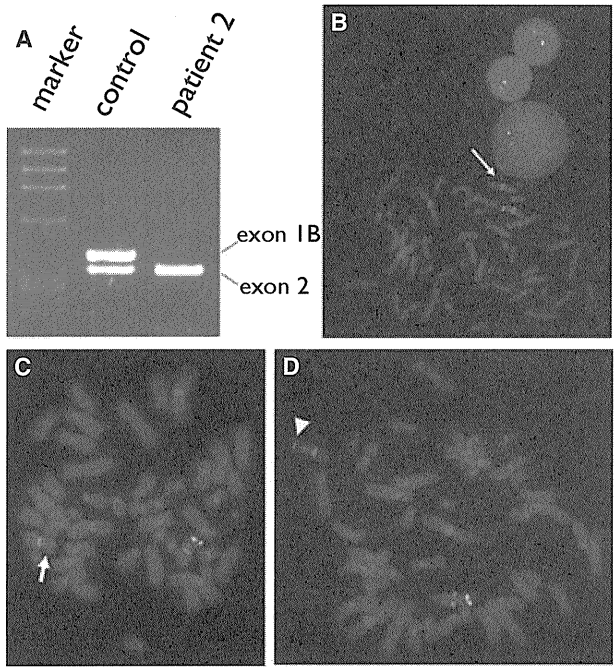

Figure 2.
Validations of genomic copy number aberrations. (**A**) Multiplex PCR amplification indicates deletion of exon 1B in Patient 1. The marker lane shows HaeIII digested φX174 DNA. (**B**, **C**) FISH analysis indicates loss of the green signal on one of the X chromosomes (arrows). For Patient 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.

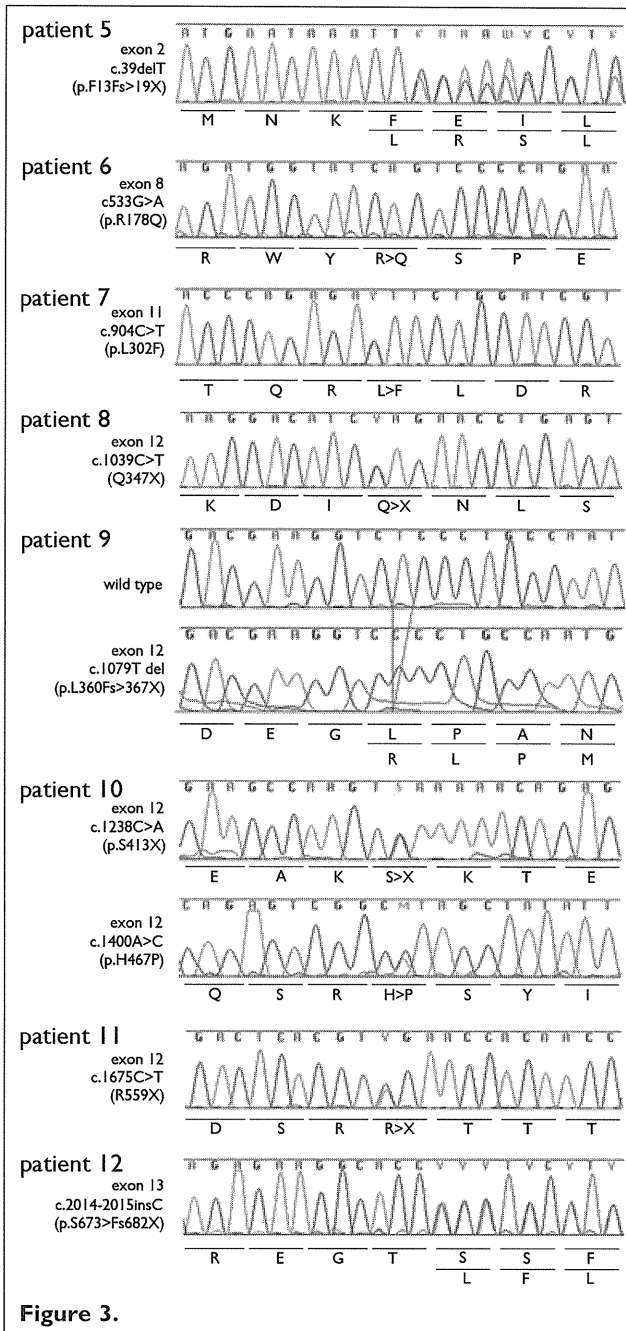

Figure 3.
Electrophoresis of the direct sequencing. Alphabetic symbols indicate amino acids. For Patients 5, 9, and 12, lines above the sequences indicate reference amino acid sequences, and lines below the sequences indicate amino acid changes caused by the mutations.

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

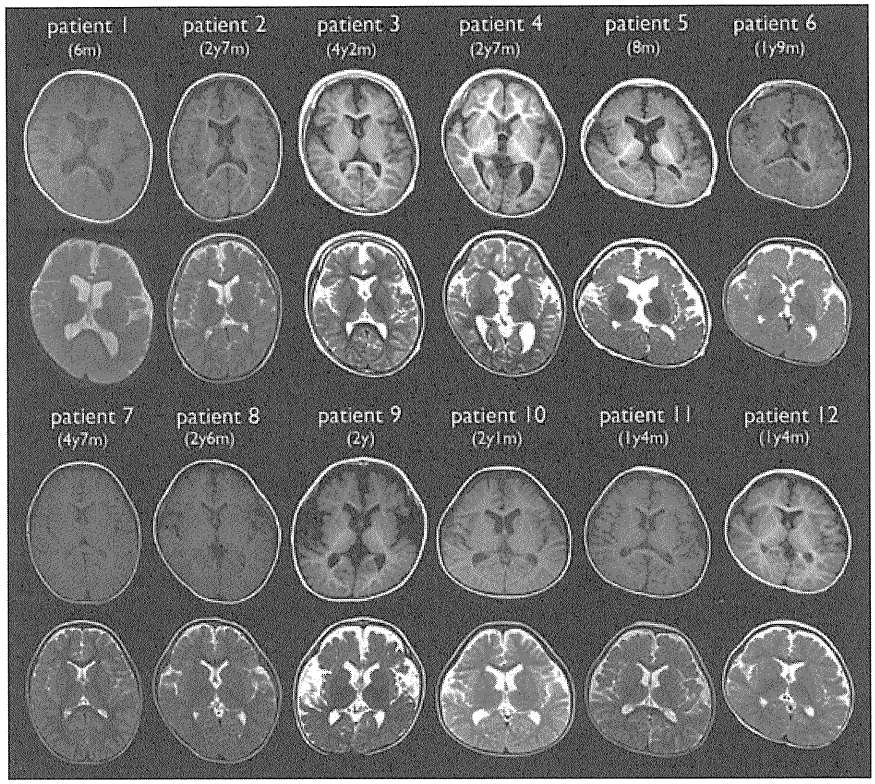

Figure 4.
Brain MRI findings of the patients. T_1- (up) and T_2-weighted (bottom) MRI indicates frontal atrophies in many patients, except for Patient 7. In Patient 6, spoiled gradient echo (SPGR) is shown instead of T_1.
Epilepsia © ILAE

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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神奈川県における先天性中枢性甲状腺機能低下症の疫学的調査　第一報

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【要　旨】

神奈川県では，濾紙血中のTSHおよびFree T4を全例で同時測定し，中枢性の先天性甲状腺機能低下症（congenital hypothyroidism, CH）もマススクリーニング対象疾患に含めている．現行のFree T4カットオフ（CO）値［0.70ng/dL］の妥当性の検討を最終目的として，県下の中枢性CHの疫学調査を立案した．今回はその一次調査結果を報告する．過去10年間の出生児を対象とした調査により，42例の中枢性CH症例が集積され，発生頻度は1：20,714人と算出された．中枢性CH以外の下垂体機能低下を伴うものが22例（52.4%）で，甲状腺機能低下のみの症例が20例（47.6%）であった．本県でマススクリーニングをうけた39例中17例（43.6%）はマススクリーニングで異常を指摘されていなかったことから，現行のCO値では，中枢性CHの約半数が見逃されている可能性が示唆された．

【キーワード】

新生児マススクリーニング，先天性甲状腺機能低下症，中枢性甲状腺機能低下症，Free T4，カットオフ値

【緒　言】

先天性甲状腺機能低下症（congenital hypothyroidism, 以下CH）は，早期発見・早期治療により正常な発育・発達が期待される疾患であり，本邦でも1979年より新生児マススクリーニング対象疾患に加えられている．スクリーニング法として，国内の多くの検査施設で採用されているのは，TSH単独測定方式，またはprimary TSH/back up FT4方式（全例にTSHを測定し，高値検体についてのみ遊離サイロキシン（Free T4, 以下FT4）を測定する）である．これらTSH測定を優先する方法は，偽陽性が少なくかつ軽症のCHを発見できる利点を有するが，サイロキシンまたはFT4優先方式と異なり，中枢性CHおよびTSH遅発上昇型CHは見逃される[1)]．この両方式の利点を生かすため，本県を含む一部の地域（札幌市，山形県，四国地方など）では，TSH・FT4を全例で同時に測定している．煩雑でかつ経費の増加も不可避であるが，米国小児科学会（American Academy of Pediatrics）の勧告では，TSH・FT4同時測定が最も理想的なCHマススクリーニング法であると記載されている[2)]．

本県でのFT4カットオフ（CO）値は0.70ng/dL（血清表示）に設定されているが，感度の向上のために1.0ng/dL程度にすべきであるとの指摘がある[3)]．しかし，CO値の引き上げにより偽陽性率も当然増加するため，陽性的中率（positive predictive value）の変動の予測が重要である．われわれは，FT4のCO値を再検討することを目的とし，その基礎調査として，県下の中枢性

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CH疫学調査を立案・実施した．今回は，その一次調査結果につき報告する．

【方　法】

2008年10月に本県内の小児科を標榜するすべての病院と療養病床を有する診療所，合計139施設に対し「先天性中枢性甲状腺機能低下症（中枢性クレチン症）の概数調査」質問用紙を郵送した．調査項目として，①調査時点でのCH診療の有無　②過去10年（1997年4月以降）のCH患児の総数　③同中枢性CH患児の総数の記載を求めた．中枢性CHの患児が存在する場合は，マススクリーニング陽性であった症例，すなわちFT4低値が指摘された症例と指摘されなかった症例の内訳，および甲状腺機能低下のみの症例と他の下垂体機能低下を伴う症例（汎下垂体機能低下症）の内訳につき質問した．また，マススクリーニングの際のFT4値が明らかな場合には，その記載を求めた．

中枢性CHの定義は，①視床下部性または下垂体性の甲状腺機能低下症　②脳腫瘍や頭部外傷など明らかに後天性の原因があるものは除く　③低出生体重児の低(F)T4血症は除く，以上の3項目を満たすものとした．甲状腺機能のみ低下している症例に限らず，下垂体機能低下を合併する症例（汎下垂体機能低下症症例）も対象としているため，口蓋裂に伴う汎下垂体機能低下症や，septo-optic dysplasiaも含んで中枢性CHと定義した．

本県のCHマススクリーニング方法は図1の通りである．すなわち，一次検査でTSHおよびFT4を測定し，TSH 30.0μIU/mL以上（血清表示）の場合は直ちに要精密検査となる．また，TSH 15.0μIU/mL以上またはFT4 0.70ng/dL未満では再採血を行い，再度TSH 15.0μIU/mL以上またはFT4 0.70ng/dL未満を示した場合に要精密検査となるシステムである．精査施設のほとんどは，小児内分泌を専門とする医師の在籍する施設である．

【結　果】

1．回収率

調査票を郵送した139施設のうち94施設より回答を得たが，うち14施設は小児科の診療を行っていないとの回答であった．これらを除外し，実際に小児科の診療が行われていた125施設に限定すると，80施設から回答が得られたこととなり，回収率は64.0%と算出された．

2．CH診療の有無と各施設別症例数（図2-a）

CHの診療を行っていると回答した施設は

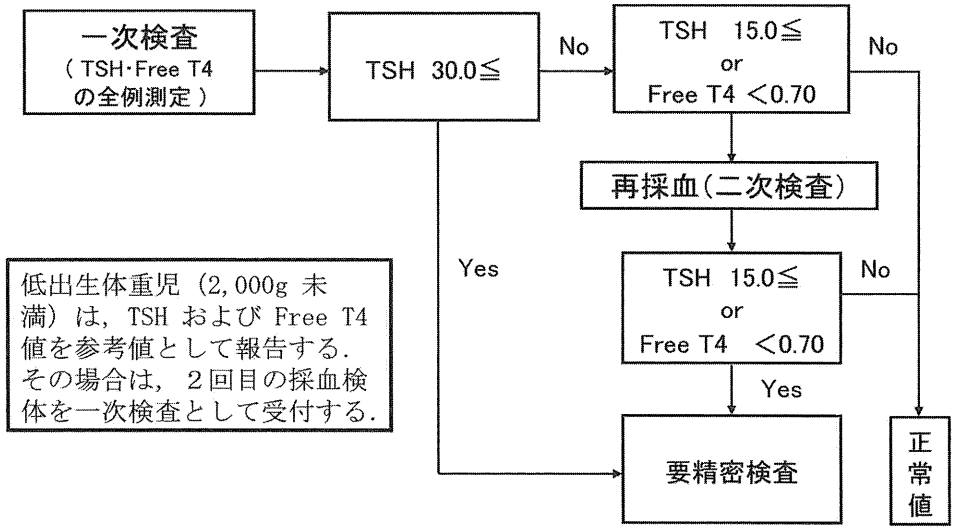

図1．神奈川県におけるCHスクリーニング方法
単位；TSHはμU/mL，Free T4はng/dL．いずれも血清表示．

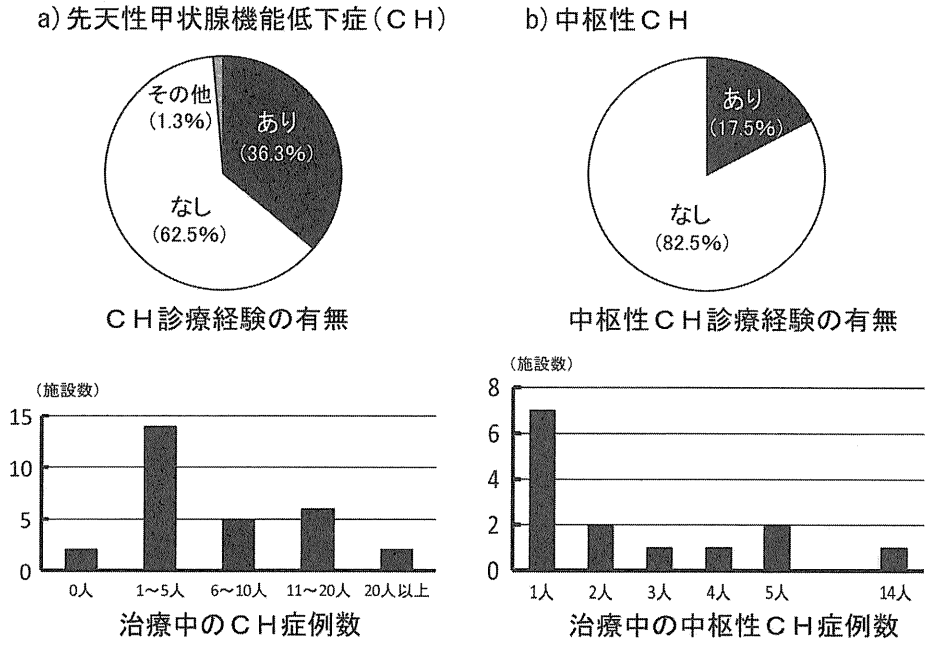

図2．診療施設別の，先天性甲状腺機能低下症（CH）の診療経験および診療症例数
a）CH全体に関する調査結果．上段の「その他」は，診断のみ行った1施設．
b）中枢性CHに関する調査結果．

36.3%（29/80施設）であり，1施設を除き小児内分泌を専門とする医師の在籍する施設であった．また，別の1施設は，診断のみ行い，治療は他院で行っているとの回答であった．治療を行っている28施設でのCH症例総数は314例となった．施設別では，症例数が5以下の施設が全体の55.2%（16/29）であった．

3．中枢性CHの診療の有無と各施設別症例数（図2-b）

中枢性CHの診療経験のある施設は17.5%（14/80施設）であり，1施設を除いた13施設は小児内分泌を専門とする医師の在籍する施設であった（施設内訳：市中総合病院 9，大学病院 4，こども病院 1）．14施設での合計症例数は42例であった．施設別の診断症例数は，1施設を除き5例以下であった（中央値は1.5名）．中枢性CH症例の詳細（中枢性CH以外の下垂体機能低下の合併の有無，およびマススクリーニングでの異常の有無）を表1に示した．下垂体機能低下を伴う症例が52.4%（22/42）で，甲状腺機能低下のみの症例が47.6%（20/42）であった．3例は他県で出生した症例であったためFT4を含むマススクリーニングは受けていなかった．本県でマススクリーニングを受け，FT4低値を指摘された症例は56.4%（22/39）であった．

4．CHと中枢性CHの本県における発生頻度

今回の調査結果と当該期間の県内出生数より，CHおよび中枢性CHの発生頻度を算出した．当該期間の本県内の出生数は807,855人であった．同期間のCH症例数は314例であり，ここから算出されるCHの発生頻度は1：2,573人となった．中枢性CHのうち本県内出生の症例数は39例であり，中枢性CHの発生頻度は1：20,714人と算出された．ただし，いずれも精査の有無やその詳細な結果は未調査であり，あくまでも一次調査での申告に基づくものである．

5．中枢性CHのマススクリーニング結果

中枢性CH42症例のうち，マススクリーニング時のFT4値が判明した12症例について，その詳細を表2に示した．FT4が0.7ng/dL以上の値であったため異常なしと判断された症例は4例（症例9～12）であった．

表1．中枢性甲状腺機能低下症の42例の病型とマススクリーニングの結果

	MS陽性	MS陰性	県外出生	
汎下垂体機能低下	7例	13例	2例	22例 (52.4%)
甲状腺機能低下のみ	15例	4例	1例	20例 (47.6%)
合計	22例 (県内出生の56.4%)	17例 (県内出生の43.6%)	3例	42例

MS：マススクリーニング

【考　察】

今回の検討において，調査用紙の回収率は64.0%と良好な成績であった．神奈川県医師会先天性代謝異常対策委員会の集計では，当該期間中にマススクリーニングで中枢性CHと診断された症例は21例であった[4]．今回の調査でマススクリーニング陽性であった中枢性CHはほぼ同数の22例であり，この点から，本調査における中枢性CHの捕捉率は良好であったと推察される．しかし，今回集積された症例は，中枢性CHが疑われる症例というべきであり，休薬後の病型診断や下垂体機能全般に関する詳細な精査結果は未調査である．したがって，以下の推論も，あくまで一次調査の結果から得られたデータにもとずくものであることを理解しなければならない．

まとまった数のCH症例を診療している施設は少なく，特に中枢性CHについては1症例のみと答えた施設が最も多かった．したがって，症例の治療方針やその経過の検討のためには，各医療機関の情報共有と連携が必要と考えられた．

今回の調査において，中枢性CHは42例が集積されたが，中枢性CH以外の下垂体機能低下症を伴うもの（汎下垂体機能低下症）と甲状腺機能低下症のみの症例が，各々52.4%，47.6%と拮抗していたことが特徴的であった．1994～1996年にオランダで行われたCHマススクリーニングの前方視的検討では，中枢性CHと診断された19例のうち78.0%が汎下垂体機能低下症であった[5]．また，2000～2004年の札幌市での検討では，中枢性CHと診断された6例のうち4例（66.7%）が他の下垂体機能低下を伴っていた[6]．さらに，1990～2000年に米国インディアナ大学で行われた後方視的研究では，中枢性CHの41/42例（97.6%）が複合型下垂体機能低下症であったとされている[7]．これらの報告では，中枢性CHは全てマススクリーニングで発見された症例に限られている．したがって，本調査結果と併せて考えると，マススクリーニングで発見されず，その後中枢性CHと診断される症例のなかに，甲状腺機能低下症のみの症例が多く存在する可能性が示唆される．しかし前述のように，今後の二次調査を経て，さらに詳細な検討を加えることが重要であろう．

当該期間の出生数から算出したCHの発生頻度は1：2,573人であった．北米・欧州での発生頻度は，3,000～4,000出生に1人とされている[2]．一方本邦では，実際にCHとして治療されている患者数は1：2,000～2,500であるとの推定がなされており，今回の検討結果とよく一致した[8]．今回の検討は，マススクリーニングを受けた直後（新生児～乳児期）の調査ではないので，一過性の甲状腺機能低下がある程度除外された，より真実に近い治療実態を反映しているものと考えられる．ただし，全例で詳細な病型診断が施行されているとは考え難く，一過性甲状腺機能低下症例も含まれていることが予測され，真のCH頻度は1：2,573人より低い値であると推測される．

中枢性CHの発生頻度は，1：20,714人と算出された．以前は本邦・欧米ともに，約100,000出生に1名程度と見積もられていたが，その後の検討により実際の発生頻度はかなり高いことが明らかにされてきた．TSH・FT4同時測定（FT4CO値1.0ng/dL）を実施している札幌市では，中枢性CHの発生頻度（2000～2004年）は，1：

13,814と報告されている[6]．オランダではprimary T4/back up TSHシステムに，さらにTBGの測定を加え（T4 -1.6SD未満，T4 SDS/TBG nmol/L 8.5未満），中枢性CH発見のための精力的な努力を行っているが，2006年の報告では中枢性CHの発生頻度は1：21,000とされている[9]．二次調査により，更に精度のよい発生頻度の把握が期待される．

本調査において中枢性CH：全CHの割合は，1：8.5であった．中枢性CH：全CHの割合は，過去の札幌市の報告では1：7.8[6]，オランダの報告では1：12.7[9]であった．本調査結果は，過去の報告と概ね同様の結果であったと考えられる．

本県で出生しFT4を含むマススクリーニングを受けた中枢性CH39例のうち，17例（43.6%）はマススクリーニング結果が正常であり，中枢性CHの約半数が見逃されている可能性が考えられた．FT4_1.0ng/dLをCO値に設定している札幌市では，FT4再検率は0.76%，FT4精査率は0.026%，陽性的中率（positive predictive value）は27.3%（文献6）より算出）であると報告されている．一方，FT4 0.7ng/dLをCO値としている本県のFT4再検率は0.16%，FT4精査率（概算）は0.013%，PPVは22%であった（2007年度実績）．仮に札幌市と同じFT4 1.0ng/dLをCO値とした場合には，再検率は0.60%に上昇すると予測された．FT4値は在胎週数や出生時体重と相関するため[10][11]，本県では体重2,000g未満の低出生体重児の場合は，生後すぐのFT4値は参考として報告するに留め，生後1ヶ月前後での再採血検体を一次検査としている（図1）．このことが，予想再検率が札幌市の値よりも低くなっている一因かもしれない．二次調査にて各症例の濾紙血FT4値が把握できれば，CO値を引き上げた場合のPPVが予測できるため，適正なCO値に関する科学的な検討が可能となると期待される．実際に，本調査でMS結果が判明した症例ではCO値1.0ng/dLとしても依然2例はMSで発見困難（表2，症例9, 12）であった．また表2に示すように，FT4は初回の採血時に比して再採血時に高値に推移する傾向が窺われた．中枢性CH児が再採血で正常と判定されることを防ぐ目的で，初回と再採血時のCO値を別個に設定する（再採血時のCO値を高く設定する）ことの必要性も示唆された．

【追　記】

春木英一先生は，平成22年4月に御逝去なさいました．先生の新生児マススクリーニング事業に対する多大なる功績に対し衷心より敬意を

表2．マススクリーニングの結果が確認できた中枢性CHの12例

症例	1回目		2回目		MS結果	病型
	TSH	FT4	TSH	FT4		
1	3.5	0.37	3.1	0.62	陽性	汎下垂体機能低下
2	3.1	0.48	1.8	0.50	陽性	汎下垂体機能低下
3	4.1	0.55	4.7	0.38	陽性	汎下垂体機能低下
4	3.0	0.68	1.3	0.68	陽性	甲状腺機能低下のみ
5	1.5	0.50	5.0	0.60	陽性	甲状腺機能低下のみ
6	4.3	0.33	3.0	0.66	陽性	甲状腺機能低下のみ
7	5.9	0.14	2.4	0.48	陽性	甲状腺機能低下のみ
8	4.6	0.43	1.1	0.66	陽性	甲状腺機能低下のみ
9	0.7	0.27	0.6	1.10	異常なし	甲状腺機能低下のみ
10	5.6	0.83	−	−	異常なし	汎下垂体機能低下
11	3.0	0.90	−	−	異常なし	汎下垂体機能低下
12	0.6	1.44	−	−	異常なし	汎下垂体機能低下

FT4：Free T4
単位；TSHはμU/mL，Free T4はng/dL．いずれも血清表示．

表し，ここに謹んでご冥福をお祈り申し上げます．

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