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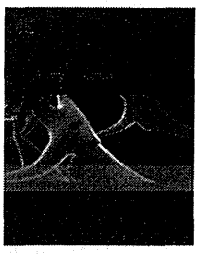

PCDH19-related female-limited epilepsy: Further details regarding early clinical features and therapeutic efficacy

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Summary Abnormalities in the protocadherin 19 (*PCDH19*) gene cause early-onset epilepsy exclusively in females. We aimed to explore the genetic and clinical characteristics of *PCDH19*-related epilepsy by focusing on its early features and treatment efficacy.
PCDH19 was analyzed in 159 Japanese female patients with early-onset epilepsy *via* direct sequencing and multiplex ligation-dependent probe amplification (MLPA) analysis.
We identified 17 patients with *PCDH19* abnormalities: point mutations were observed in 14 patients and whole *PCDH19* deletions were detected in 3 patients. One affected sister of a proband with a mild phenotype was also analyzed. The frequency of *PCDH19* deletion among all probands identified in Japan was 12.5% (3/24, including 7 probands reported previously by us). Clinical features included early onset (mean age at onset, 8.6 months), recurrent clusters of brief seizures (17/18), fever sensitivity (18/18), tonic seizures (13/18, probably including focal tonic seizures), tonic–clonic seizures (8/18), focal seizures often with subsequent generalization (17/18), intellectual disabilities (15/18), and autistic traits (13/18). Three patients exhibited delay in motor milestones before seizure onset. In 16 patients, seizures appeared in clusters from the onset of the disease. Among 6 patients for whom detailed information at onset was available, 2 onset patterns were identified: a biphasic course of short seizure clusters (each within days) in 2 patients and a prolonged course of clusters (from weeks to a month) in 4 patients. In both cases, initial seizures started during fever and transiently disappeared with the decline of fever; however, afebrile clusters recurred. In the former patients, motor development was delayed before onset, and seizures appeared in strong clusters from the onset of the disease. In the latter patients, initial development was normal and initial seizures were mild, but were followed by strong clusters lasting several weeks, even without fever. Treatment using phenytoin, potassium bromide, and clobazam showed high efficacy. Although focal seizures were the main feature in *PCDH19*-epilepsy, the efficacy of carbamazepine was poor.
This study highlighted the significance of *PCDH19* deletion, a unique pattern of initial seizure clusters, and the efficacy of antiepileptic drugs. Our data will facilitate early diagnosis and development of a treatment strategy for better clinical management of patients with *PCDH19*-related epilepsy.

Introduction

Protocadherin 19 (*PCDH19*)-related female-limited epilepsy (*PCDH19*-epilepsy) is a unique X-linked disease, in which males with *PCDH19* mutation are unaffected (Dibbens et al., 2008; Juberg and Hellman, 1971; Ryan et al., 1997; Scheffer et al., 2008). A significant number of patients with this epilepsy have been identified in the past years, and its significance has been increasing rapidly (Depienne et al., 2009, 2011; Marini et al., 2010; Specchio et al., 2011). *PCDH19* encodes protocadherin 19 and is located on chromosome Xq22.1 (Vanhalst et al., 2005). Most of the pathogenic mutations of *PCDH19* occur in exon 1, and truncation mutations have been identified exclusively in downstream exons, with the exception of exon 2 (Depienne and LeGuern, 2012). A microdeletion involving *PCDH19* has also been reported in 6 patients (Depienne et al., 2009, 2011; Vincent et al., 2012). The lack of "cellular interference" has been hypothesized as the mechanism underlying the male sparing observed in this disease (Depienne et al., 2009): in females, one of the X chromosomes is inactivated, which results in somatic mosaicism regarding PCDH19 expression—wild type *vs.* mutant or none (in the case of nonsense-mediated mRNA decay (Dibbens et al., 2008)). This mosaic status may be necessary for the pathogenesis of *PCDH19*-epilepsy, which is termed as "cellular interference".

The clinical characteristics of *PCDH19*-epilepsy have been well described (Depienne and LeGuern, 2012; Higurashi et al., 2012; Scheffer et al., 2008; Specchio et al., 2011). These include early seizure onset, fever sensitivity, seizure clusters of brief seizures—especially focal seizures, often with fearful screaming (Marini et al., 2012)—frequently associated with fever, varying degree of intellectual disability, and autistic traits. *PCDH19* mutations were initially discovered in large families (Dibbens et al., 2008) and in female patients with a Dravet syndrome (DS)-like phenotype who lacked the *SCN1A* mutation (Depienne et al., 2009; Marini et al., 2010); however, it is now well known that only a portion of patients manifest a phenotype resembling DS (Depienne and LeGuern, 2012; Higurashi et al., 2012; Marini et al., 2012). *PCDH19*-epilepsy rarely exhibits prolonged seizures, photosensitivity, or clonic, myoclonic and absence seizures. In its long-term course, seizure cessation is common after school age (Higurashi et al., 2012; Scheffer et al., 2008), and most patients achieve good motor skills and walk independently without ataxia. Accordingly, it is of critical importance to differentiate this clinical entity from DS and establish an early diagnosis of *PCDH19*-epilepsy to provide better clinical management to these patients.

For this purpose, we performed an additional genetic analysis study of *PCDH19* in Japanese female patients with early-onset epilepsy, including screening for *PCDH19* deletion *via* multiplex ligation-dependent probe amplification (MLPA) analysis, and explored the early clinical details of newly identified patients with *PCDH19*-epilepsy. Here we present the significance of *PCDH19* deletion in this disease and distinct patterns of initial seizures, which will facilitate the early diagnosis of this disease. We also summarize the efficacy of antiepileptic drugs in our patients in an attempt to identify an appropriate treatment strategy.

Subjects and methods

Subjects and clinical information

One hundred and fifty-nine female patients with various types of epilepsy were newly enrolled in this study. These individuals were referred to our laboratory for *SCN1A* and/or *PCDH19* analyses because of their clinical features, such as early seizure onset, fever sensitivity, status epilepticus, seizure clusters, intractability of seizures, and concomitant intellectual disability, and included 53 patients with DS and 67 patients with symptomatic focal epilepsy. *SCN1A* abnormalities were screened before this study and were identified in 45 patients.

The clinical information was collected using a questionnaire and by directly contacting the physicians in charge: they were all pediatric neurology specialists and were well experienced in epilepsy care. The presence of "autistic traits" in patients was determined *via* medical interviews and observation of the patient's behavior, to determine weather they exhibited impairment in one or more of the 3 items included in DSM-IV-TR criteria for autism (American Psychiatric Association, 2000). To assess the treatment efficacy of antiepileptic drugs, the reduction of the rate of seizure frequency was determined by comparing the seizure records of periods of at least 6 months before and after the beginning of drug administration, and was classified as follows: excellent, ≥90% reduction; effective, ≥50% reduction; ineffective, <50% reduction; and exacerbation, seizures increase. Written informed consent for genetic analysis was obtained from the patients' guardians before blood sampling. This study was approved by the Human Ethics Committee of Fukuoka University (Approval No. 09-09-05).

Genetic analysis of *PCDH19*

Direct sequencing was first performed as described previously (Higurashi et al., 2012). In the present study, MLPA analysis was also performed to detect *PCDH19* deletion. Because the MLPA analysis was not performed in our previous study, the previous cohort (116 patients) was included in this analysis (Higurashi et al., 2012); however, no abnormalities were identified in that cohort. The probes used for MLPA reaction were designed to hybridize to each region of the 6 exons. The details of the MLPA probes and analytical methods, and the protocols used in the other analyses performed in this study, including fluorescence *in situ* hybridization (FISH) for *PCDH19*, array comparative genomic hybridization (aCGH), and determination of X-chromosome inactivation status at the human androgen receptor gene (*HUMARA*) (Allen et al., 1992; Mei et al., 2010), are described in the Supplementary Methods.

Results

Abnormalities in *PCDH19*

Heterozygous *PCDH19* abnormalities were identified in 17 patients: point mutations were detected in 14 patients (Table 1, Patients 1–14; GenBank accession number of the complete human *PCDH19* mRNA, EF676096.1) and whole *PCDH19* deletions were detected in 3 patients (Table 1, Patients 15–17, Supplementary Fig. S1A and B). Four affected relatives were also identified: the younger sister of Patient 4 (Patient 4s) and the mothers of Patients 6, 12, and 14 (clinical information was available for Patient 4s). Novel missense mutations (p.R198L, p.S139L, p.D90V, p.D596V, and p.D157N) occurred at highly conserved residues (Supplementary Fig. S2). Patients 10 and 14 had a nonsense mutation in exons 2 and 4, respectively; the remaining patients had a mutation in exon 1. The exon 2 mutation detected in Patient 10 was inherited from the healthy father. Although she had a typical phenotype of *PCDH19*-epilepsy (Supplementary Information), her 2 older sisters with the same mutation were completely healthy. They all exhibited a random X-inactivation pattern (paternal:maternal, 38:62 [Patient 10] and 58:43 and 28:72 [her sisters]).

PCDH19 deletion was also confirmed by FISH analysis (Supplementary Fig. S1B) In Patient 15, aCGH revealed the presence of a 4-Mbp deletion that also involved several flanking genes (Supplementary Fig. S1C), including *SPRX2*, which is responsible for rolandic epilepsy, mental retardation, and perisylvian polymicrogyria only in males (Royer et al., 2007), and other genes related to non-neuronal diseases (Bione et al., 1998; Tolppanen et al., 2010). However, as reported previously (Depienne et al., 2011; Vincent et al., 2012), no significant differences in clinical features were identified between the 3 patients with *PCDH19* deletions and patients with point mutations (the clinical details of Patients 15 and 16 are described in the Supplementary Information and those of Patient 16 are also presented in Fig. 1C). The frequency of *PCDH19* deletion among all *PCDH19*-epilepsy cases in Japan (24 probands in total, 7 reported previously (Higurashi et al., 2012)) was 12.5% (3/24).

Overview of clinical features

The clinical information of the newly identified patients ($n=18$, including Patient 4s) is summarized in Table 1. None of these patients had any hypoxic events during the gestational and perinatal periods. Their main features were consistent with those described previously: mean (±standard deviation) age at onset of 8.6±4.2 (4–25) months; seizure clusters (17/18; Patient 4s, who had a mild case, was the exception); fever sensitivity (18/18); brief habitual seizures (duration of a few minutes or less, often ≤1 min); and low incidence of prolonged seizure (defined duration: ≥15; 2/18, Patients 4 and 9; Patient 4 exhibited this only at onset, and Patient 9 had several prolonged complex partial seizures at the age of 6 during dose reduction of oral antiepileptics). However, some patients presented with transient severe seizure clusters that were close to status epilepticus. In Patient 10, this occurred just after the cessation of continuous administration of midazolam. Although seizure frequency was generally high during early childhood (monthly to every several months), it was highly variable among patients, and Patient 9 had no seizure between 2 years and 4 years and 4 months of age followed by recurrence.

Seizure types included tonic (13/18), tonic–clonic (8/18), and focal seizures often with subsequent

Table 1 Clinical characteristics of the patients with *PCDH19*-related epilepsy.

Pt	*PCDH19* abnormality	Mutagenesis	Onset (m)	Development at onset	Initial sz pattern[a]	Sz type[a]	Sz cluster	FS focus[b]	Brain MRI
1	p.R198L c.593G>T	NA	7	N	Afebrile/FS, TC cluster	FS, T, TC	+	lt-O/7m, lt-CO/2y rt-P/4y	N
2	p.K120RfsX3 c.357delC	NA	10	N	Febrile/isolated TC, T → afebrile/T,FS cluster	T, TC, FS	+	rt-mTpT/10m	N
3	p.Y166* c.497_498insA	NA	4	N	Febrile → afebrile/TC, T cluster	T, TC	+	lt-aT-Fp/4y7m	N
4	p.D45GfsX43 c.134-135ACdel	Fa	8	N	Febrile/TC (SE) → (a)febrile/FS cluster	TC, FS	+	rt, lt-F, Te	Heterotopia lt-F
4s[c]	Same as above	Fa	22	N	Febrile/isolated T	T, FS	–	NA	N
5	p.N340S c.1019A>G	*De novo*	5	N	Febrile/FS, T cluster	FS, T	+	rt-C,Te/3y10m bil-F/7y11m	N
6	p.S139L c.416C>T	Mo	9	N	Febrile → afebrile/T, FS cluster	T, FS	+	NA	N
7	p.D90V c269A>T	*De novo*	11	N	Afebrile/FS, T cluster	FS, T	+	rt-C,O/4y3m	N
8	p.D596V c.1787A>T	Fa	5	N	Febrile/FS cluster	FS, T	+	rt-P,O,pT/5m rt-P/5y4m	Intensity laterality, O
9	p.Y366LfsX10 c.1091_1092ins C	NA	5	Delayed head control	Febrile → afebrile/FS cluster	FS, T	+	lt-mTaT/5m lt-aT/2y6m	Atrophy lt-PO
10	p.L719* c.2156T>G (Ex2)	Fa	13	N	Febrile/isolated TC × 2	TC, FS	+	rt-F/2y4m	N
11	p.S350* c.1048C>G	Fa	5	Delayed head control	Febrile/FS cluster	FS, T	+	rt, lt-F	N
12	p.N340S c.1019 A/G	Mo	8	N	Febrile → afebrile/TC, FS cluster	TC, FS	+	NA	N
13	p.R886* c.2656C>T (Ex4)	*De novo*	11	Delayed sitting/crawling	Febrile → afebrile/FS, TC cluster	FS, TC	+	NA	N
14	p.D157N c.469G>A	Mo	5	N	Febrile/T cluster	FS	+	lt-F	Atrophy rt-HIP
15	Whole deletion	*De novo*	9	N	Febrile/FS cluster	FS, T	+	lt, rt-F,P/2y9m	T2 high lesion lt-F WM
16	Whole deletion	*De novo*	10	N	Febrile/FS, T cluster	FS, T, TC	+	lt-O/11mo bil-C/1y4m	N
17	Whole deletion	*De novo*	8	N	Febrile/FS, T cluster	FS, T	+	NA	N

1	MDL (buccal, iv) fPHT	CLB ZNS	PB KBr PHT TPM VPA	6y5m	DQ 80.5 2y6m	N	Autistic
2	MDL	CLB ZNS	PB KBr LEV	4y4m	DQ 56 3y6m	N	Autistic
3	MDL (DZP sup)	KBr PB	CBZ CZP TPM	7y2m	Severe 7y	N	Autistic
4	MDL	KBr VPA	(CZP) (CBZ) (PB) (PHT)	10y10m (8y)	IQ 42 9y10m	N	Autistic
4s	NA	VPA CZP		7y2m (3y)	—	N	Hyperactive
5	MDL (DZP sup)	LTG KBr CZP	PB ZNS CLB VPA NZP B6 GBP STM *CBZ PHT*	8y	DQ 44 7y4m	N	
6	(DZP sup) (PB sup)	VPA	(CBZ) (PB)	13y9m (11y9m)	IQ 62 11y9mo	N	
7	(MDL) (DZP)	VPA PHT CZP TPM	CLB PB ZNS GBP LTG	6y7m (4y11m)	Mild ~ moderate	N	Autistic
8		CLB CBZ TPM	CZP ZNS PB *VPA*	5y8m	DQ 49 4y11m	N	Autistic
9	MDL (≥4y6m)	CBZ VPA CLB	PHT TPM (CZP) (LEV)	8y4m	IQ 51 7y4m	N	Impulsive
10	MDL (PB) mPSL	LEV	CBZ CZP VPA (LTG)	3y6m	—	N	Autistic Hyperactive
11	MDL PHT	PHT	B6 CLB VPA PB KBr CZP ZNS	7y	DQ 48 3y	N	Autistic Hyperactive
12	MDL PHT	LEV	CBZ ZNS CLB CZP TPM	5y11m	DQ 38 4y7m	Truncal instability	Autistic Hyperactive
13	(MDL) TPL PB sup/iv	CLB KBr	PB CZP (CBZ)	3y4m	DQ 76 2y3m	Mildly hypotonic	Autistic Hyperactive
14		TPM	VPA CLB (CBZ) (PB)	7y	DQ 50 1y6m	Mildly ataxic	Autistic Hyperactive
15	(DZP sup)	CZP	LEV VPA OXC CLZ	2y10m	DQ 49 2y9m	Mildly unstable gait	Autistic
16	MDL TPL (PHT)	PHT	VPA PB CBZ (LEV)	2y1m	Mild	N	Hyperactive
17	MDL	TPM CLB	VPA CBZ ZNS	4y	DQ 63 2y6mo	N	Autistic Hyperactive

Pt, patient; sz, seizure; MRI, magnetic resonance imaging; NA, not analyzed; N, normal; FS, focal seizure; lt, left; O, occipital; C, central; rt, right; P, parietal; mT, mid temporal; pT, posterior temporal; aT, anterior temporal; Fp, frontopolar; Fa, father; SE, status epilepticus; F, frontal; Te, temporal; bil, bilateral; Mo, mother; HIP, hippocampus; WM, white matter. ID, intelligent disability; MDL, midazolam; fPHT, fosphenytoin; CLB, clobazam; ZNS, zonisamide; PB, phenobarbital; KBr, potassium bromide; PHT, phenytoin; TPM, topiramate; VPA, sodium valproate; DQ, developmental quotient; N, normal; LEV, levetiracetam; DZP, diazepam; sup, suppository; CBZ, carbamazepine; CZP, clonazepam; LTG, lamotrigine; NZP, nitrazepam; B6, vitamin B6; GBP, gabapentin; STM, sultiam; IQ, intelligent quotient; OXC, oxcarbazepine; CLZ, clorazepate; TPL, sodium thiopental.

[a] Tonic (T) indicates generalized, secondary generalized, or focal tonic seizures, and tonic–clonic (TC) indicates generalized or secondary generalized seizures.

[b] Determined by ictal recordings of electroencepharography.

[c] Younger sister of Patient 4.

[d] Administered intravenously otherwise specified, and drugs in parenthesis indicates "effective in some degree".

[e] Underline indicates "excellent" efficacy.

[f] Italics indicates "exacerbation", and drugs in parenthesis "undetermined" efficacy.

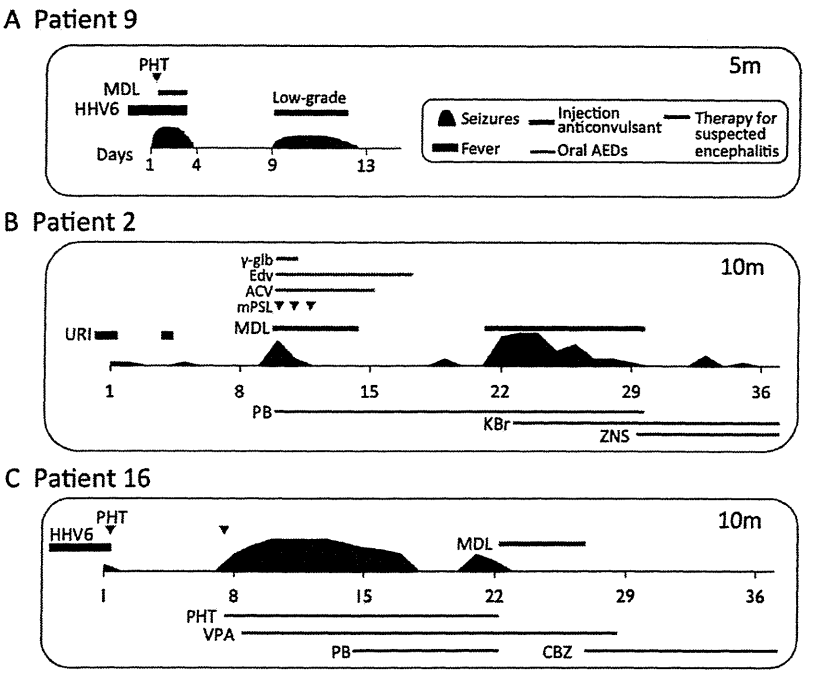

Figure 1 Examples of biphasic short seizure clusters and prolonged seizure clusters during the early phase. The early clinical courses of Patients 9 (A), 2 (B), and 16 (C) are shown. (A) Brief focal seizures consisting of motion arrest, blank eye, and facial cyanosis were repeated 33 times over 3 days during fever of exanthema subitum type and disappeared with the decline of fever. However, 5 days later, seizures accompanied by low-grade fever recurred and were repeated 26 times over 4 days. (B and C) Patients 2 and 16 had seizure onset at 10 months of age. In the initial episodes, seizures started in a mild fashion with some delay (2nd and 4th day, respectively) from the appearance of fever. Although seizures transiently disappeared with the decline of the fever, they reappeared in strong clusters without fever. (B) Brief isolated generalized convulsions associated with fever appeared on days 1, 2, and 5. Strong clusters of brief convulsions and focal seizures, often with secondary generalization, appeared on day 9; treatments for suspected acute encephalitis were administered. Although intermittently, seizure clusters were repeated over a total period of 1 month. The patient had no seizures until recurrence at the age of 2 years and 4 months. (C) The initial seizures appeared in mild clusters. ACV, acyclovir; AEDs, antiepileptic drugs; CBZ, carbamazepine; Edv, edaravone; γ-glb, gamma globulin; HHV6, human herpesvirus 6; KBr, potassium bromide; MDL, midazolam; mPSL, methylpredonisolone; PB, phenobarbital; PHT, phenytoin; URI, upper respiratory infection; VPA, sodium valproate; ZNS, zonisamide.

generalization (17/18). Focal seizures appeared from the onset of the disease in 14 patients. Here, "tonic seizure" may include focal tonic seizures. In generalized convulsions, clonic component was often less represented. The frequent symptoms of focal seizures included motion arrest, cyanosis, eye deviation, respiratory alteration, systemic jerks, mild clonic, complex movement in the face and/or extremities, and tonic symptoms. In our cases, seizures confirmed by ictal electroencephalography (EEG) included only focal seizures, and ictal activities often originated in the frontal and/or temporal regions (9/13). A posterior focus involving the occipital region was observed in 4 cases, especially during infancy. None of our patients had myoclonic and absence seizures. Patient 15 had a very brief focal seizure with momentary jerks of the trunk and limbs during sleep, independently or as the initial symptom of her longer seizure (Supplementary Video and Supplementary Fig. S3). This seizure seemingly mimicked a myoclonic seizure, which is difficult to distinguish without video-EEG recordings.

Frequent abnormalities in interictal EEG included focal (poly)spike(s)/spike(s)-and-wave discharges (14/18) and slowing of the background and basic activities (4/18). Patient 6 showed irregular spike-and-wave discharges evoked by 10–20 Hz of photic stimulation and diffuse sharp-and-wave discharges during sleep at age 11; these findings were not observed in her earlier examinations. Mild abnormalities in brain MRI were identified in 5 patients; a series of examinations revealed that those lesions were not the main cause of their epilepsy.

A mild delay in motor milestones before seizure onset was noted in 3/18 patients; their subsequent motor development was good, and all patients with the exception of Patient 12 walked independently without significant motor impairment at the time of this study. Intellectual disability [15/18 (7 mild, 7 moderate, and 1 severe)], autistic traits (13/18), and hyperactive behavior (8/18) were commonly observed. The autistic symptoms of our patients included problems in eye contact, language skills, eating, and appropriateness of behavior.

Patterns of initial seizures

Seizure clusters were highly consistent in *PCDH19*-epilepsy and appeared throughout the clinical course of patients,

mostly from the onset of seizure (16/17, Patient 10, who had a simple febrile seizure at onset, was the exception). The detailed clinical course of the initial seizure clusters could be obtained in 6 patients, and 2 patterns of seizure occurrence were identified. The first pattern was characterized by the biphasic appearance of short seizure clusters and was observed in 2 patients (Patients 9 and 13, Fig. 1A): the earlier phase comprised strong seizure clusters during fever, which disappeared with the decline of the fever. However, seizure clusters recurred within several days without fever and remitted within days. Interestingly, both patients had motor delay since before the onset of seizure.

The second pattern was characterized by seizure clusters that intensively repeated for weeks to 1 month. This pattern was confirmed in 4 patients (Patients 2, 4, 12, and 16), who all had normal psychomotor development before the onset of seizure. The clinical course of Patients 2 and 16 are presented in Fig. 2B and C, respectively. In this pattern, several mild seizures appeared during fever. Although seizures disappeared with the decline of the fever, strong seizure clusters started without fever and continued intermittently over weeks. More specifically, Patients 2 and 4 were suspected of having some acute encephalitis/encephalopathy and their treatment included immunotherapy. However, each seizure was brief and the interictal consciousness was basically clear. After the remission of the prolonged clusters, seizures disappeared for months or longer, until the next recurrence.

Treatment of seizures

During the acute phase, the efficacy of continuous administration of midazolam in suppressing the ongoing seizure clusters was confirmed in 13 patients, often at a lower dosage (≤0.2 mg/kg/h). Nevertheless, its efficacy was often insufficient to manage strong clusters during early childhood. Even when seizures were controlled by midazolam, dose reduction and discontinuation soon resulted in seizure recurrence, and sometimes even in seizure aggravation as described above (Patient 10). Intravenous administration of phenytoin/fosphenytoin or phenobarbital was also effective in 6 cases, although often transiently. Interestingly, in Patient 10, the administration of methylprednisolone (10–30 mg/kg/day for 3 days) successfully stopped ongoing clusters at several occasions at age 2. Patient 2 also exhibited transient seizure disappearance after methylprednisolone administration for suspected encephalitis at age 10 months (Fig. 1B).

The prophylactic efficacy of antiepileptic drugs, which were administered to 8 or more patients, is summarized in Fig. 2. This summary also includes information on our 8 patients who were reported previously ($n=25$ in total) (Higurashi et al., 2012). Phenytoin, potassium bromide, and clobazam were often beneficial for decreasing seizures. However, no definitive drug that prevented further seizures in multiple patients was identified. Carbamazepine had the poorest efficacy among the drugs recorded. Other drugs used by a small number of our patients included levetiracetam (number of patients with excellent/effective/ineffective efficacy, 0/2/2), clorazepate (1/1/1), lamotrigine (0/1/1), gabapentin (0/0/2), vitamin B6 (0/0/2), nitrazepam (0/0/1), sultiame (0/0/1), oxcarbazepine (0/0/1), and acetazolamide (0/1/0). None of our patients received oral corticosteroids, adrenocorticotropic hormone therapy, ketogenic diet, or vagal nerve stimulation.

Discussion

In this study, we found that 12.5% of Japanese probands with *PCDH19*-epilepsy carried a *PCDH19* deletion, which clearly indicates the significance of screening for this deletion in cases without point mutations. We also identified a nonsense mutation in exon 2 of *PCDH19* in a patient with a typical phenotype of *PCDH19*-epilepsy (Patient 10). Although one mutation can be associated with a significant phenotypic variation among patients (Higurashi et al., 2012), her sisters with the same mutation were completely healthy. The presence of splicing variants lacking exon 2 (Dibbens et al., 2008) may not adequately account for the differences in disease development observed among these sisters, and some triggering factors other than *PCDH19* mutation might be involved in the pathogenesis.

In our patients, 2 distinct patterns of seizure onset were identified: a biphasic course of relatively short seizure clusters (each lasting up to a few days), and a prolonged course of seizure clusters (intermittently repeated for weeks or 1 month). In both cases, initial seizures remitted with the decline of fever. In the patients with the former pattern, initial seizures appeared in strong clusters, and their motor developmental retardation started before the onset of seizure. In contrast, in the patients with the latter pattern, the development was normal at the onset of symptoms and initial seizures were mild, but were followed by strong seizure clusters lasting for weeks without fever. These findings let us to speculate that, in the former pattern, some pathological conditions had already progressed before seizure onset that induced strong clusters from seizure onset. However, in the latter pattern, such pathological conditions might gradually emerge around the onset of seizures that induce subsequent strong clusters. Considering the case in which steroid administration was strikingly effective in suppressing seizure clusters, the inflammatory process may be one possible modifier of such clinical variations. Further biological studies are necessary to address these issues.

Such prolonged seizure clusters, which are reminiscent of a type of acute encephalitis/encephalopathy, may be a key feature that suggests the presence of *PCDH19*-epilepsy, and genetic analysis should be considered in such cases. Human herpes virus 6 and the influenza virus were often the pathogen underlying the fever observed at the onset of seizures in our patients and cause acute encephalopathy, such as that presenting with biphasic seizures and late reduced diffusion (Takanashi et al., 2006). Although it exhibits a higher average age at onset, febrile infection-related epilepsy syndrome is also a peculiar form of childhood-onset epilepsy that presents with prolonged courses of seizure clusters, often evolving into status epilepticus (van Baalen et al., 2010). In our patients, status epilepticus, impairment of interictal consciousness, leukocytosis in the cerebrospinal fluid, and brain MRI

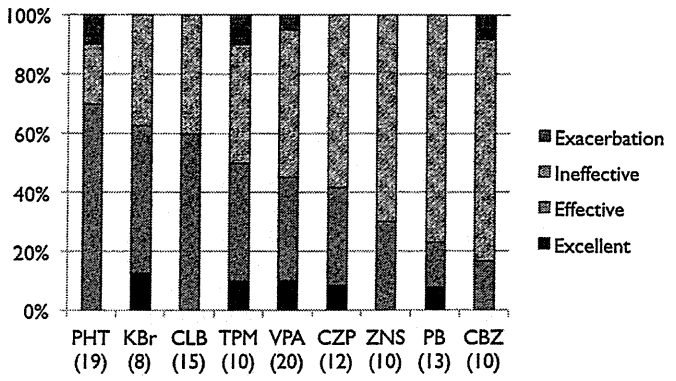

Figure 2 Prophylactic efficacy of antiepileptic drugs. Drugs administered to 8 or more patients (excluding cases of undetermined efficacy) are shown. The numbers in parenthesis indicate the number of patients assessed.

abnormalities characteristic of each type of encephalitis were rare and may be used to distinguish these diseases.

The evaluation of the prophylactic efficacy of anticonvulsants is challenging, because seizure frequency fluctuates markedly and depends significantly on the incidence of febrile illnesses and age. Such possible influences should be considered for the interpretation of the data presented in Fig. 2, and a more rigorous evaluation will be necessary in the future. However, an outline of efficacy, at least, can be speculated from our data. Although patients with *PCDH19*-epilepsy exhibited mainly focal seizures, the low efficacy of carbamazepine should be noted. The profile of drugs that showed higher efficacy was similar to that observed in DS, with the exception of phenytoin (Chiron, 2011). Unlike that which was observed in DS, phenytoin may be placed at a higher priority in cases with a high therapeutic need over the risk of gingival enlargement. Considering the unique pattern of seizure appearance in this disease, the addition of multiple antiepileptic drugs in rapid sequence should be avoided, even when seizure clusters continue.

This study highlights the significance of screening for *PCDH19* deletion, the characteristics of early seizures, and the efficacy of acute- and prophylactic treatment. Our data will contribute to the early diagnosis of the disease and the selection of more favorable treatments and may help avoid excessive treatment and provide better clinical management. In the next phase of research, the efficacy of antiepileptic drugs should be studied prospectively. In addition, it is also necessary to evaluate whether a better neurological outcome can be obtained by the improvement of seizure control.

Conflict of interest

All authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eplepsyres.2013.04.005.

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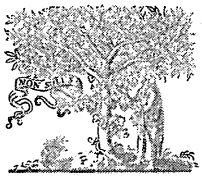

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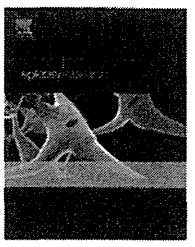

Genetic variations of immunoregulatory genes associated with Rasmussen syndrome

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Rasmussen syndrome;
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T-bet;
Epilepsy

Summary
Objective: To elucidate the genetic predisposition of Rasmussen syndrome (RS).
Methods: In 29 Japanese patients, we examined the genome sequences of *cytotoxic T-lymphocyte-associated protein 4 (CTLA4)*, *programmed cell-death 1 (PDCD1)*, and *T-bet (TBX21)* genes by direct sequencing, and evaluated the significance of SNPs (single nucleotide polymorphism) by comparison with Hap Map data.
Results: In all patients, no disease-causative mutations were found in *CTLA4*, *PDCD1*, and *T-bet*. However, rs231775 SNP in exon 1 of *CTLA4* showed significant positive genotypic ($p = 0.0363$) and allelic associations ($p = 0.0137$) with onset of RS compared with Japanese controls, as did rs231779 SNP in intron 1 of *CTLA4* ($p = 0.0467$ and 0.0188, respectively). Also, rs2227982 SNP in exon 5 of *PDCD1* showed significant positive genotypic and allelic associations with RS ($p = 0.0145$ and 0.0114, respectively). Poor cognitive outcome (IQ below 50) was found in 0% of wild type (C/C), 9% of heterologous (C/T) and 25% of homologous (T/T) genotype of rs2227982. Quadriplegia was found only in homologous (T/T) genotype, and hemiplegia was in heterologous (C/T) and homologous (T/T) genotype of rs2227982. No association between SNPs of *T-bet* and RS onset

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was found. Regarding SNPs in promoter regions (rs4794067 and rs17250932) of *T-bet*, however, IQ below 50 was found in 19% of wild type (T/T) and 0% of heterologous (T/C) genotype of rs4794067, and in 19% of wild type (T/T) and 0% of heterologous (T/C) genotype of rs17250932. Quadriplegic patients were found only in wild-type patients (rs4794067 and rs17250932).
Conclusions: We identified three SNPs (rs231775, rs231779, rs2227982) as some of the SNPs associated with onset of Japanese RS. We need further studies in other populations to confirm these genetic predispositions in RS.

Rasmussen syndrome or Rasmussen encephalitis (RS) is a slowly progressive, autoimmune neurological disease, and shows intractable epileptic seizures including epilepsia partialis continua (EPC) (Rasmussen et al., 1958; Bien et al., 2005; Takahashi, 2006). Infection occurring around two weeks prior to onset is observed in 38% of the patients (Takahashi, 2006). Histological examination usually shows inflammatory lesions with T cell infiltration. The immunopathology of RS is attributed mainly to activated cytotoxic T cells (CTLs) (Bien et al., 2002). The CSF levels of IFNγ, IL-12, and granzyme B levels are elevated in the early stage, suggesting Th1 and CTL involvement (Takahashi et al., 2009). Immunomodulatory therapies using intravenous immunoglobulin, plasmapheresis and tacrolimus have been reported to improve outcome (Bien et al., 2005; Takahashi et al., 2013). These data suggest that incomplete inhibition of CTLs activated by acute infection may contribute to the pathophysiology of RS.

Cytotoxic T-lymphocyte-associated protein 4 (CTLA4) (MIM:123890) is a negative regulator of the immune system, resulting in inhibition of activated T cells. Ctla4 knockout mice show lethal lymph-proliferative inflammation (Waterhouse et al., 1995). Gene association studies reported a strong association of polymorphism of *CTLA4* with autoimmune diseases (Ghaderi, 2011). Programmed cell-death 1 (PDCD1) (MIM:600244) is also a negative regulator of the immune system, resulting in inhibition of activated T cells. *Pdcd1* knockout mice are susceptible to autoimmune diseases (Keir et al., 2008a). These mice have regulatory T cell dysfunction, resulting in susceptibility to experimental autoimmune encephalomyelitis (Wang et al., 2010). T-bet (TBX21) (MIM:604895) promotes differentiation of naïve T cells into Th1 cells that are essential for autoimmunity (Lazarevic and Glimcher, 2011), and expression of granzyme B in $CD8^+$T cells (Ji et al., 2011). We examined the associations of disease-causative mutations and polymorphisms in these immunoregulatory genes with Japanese RS.

Patients and methods

This retrospective study was performed at the National Epilepsy Center, Japan, after obtaining approval from the ethical committee.

Patients

We identified 57 Japanese patients who presented with a diagnosis of RS and were referred to the National Epilepsy Center from all over Japan between 1991 and 2012. We reassessed the diagnosis basically according to the European diagnostic criteria for RS (Figure A1) (Bien et al., 2005). Of 57 patients, two patients who had no frequent partial seizures, and six patients who had no unihemispheric cortical dysfunction were initially excluded from a diagnosis of RS. Of the six patients without unihemispheric cortical dysfunction, five were subsequently diagnosed as having RS based on characteristic histology, elevated granzyme B in CSF, or high intensity lesion on MRI characteristic of RS (Bien et al., 2005; Yamazaki et al., 2011). Of 49 patients with unihemispheric cortical dysfunction, one patient was excluded by histological findings. From the 53 patients with a confirmed diagnosis of RS, we examined only 29 patients who were actively treated in our epilepsy center and Nishi-Niigata hospital by the experimental costs. All 29 patients gave informed consent by the methods approved by the ethical committee.

Methods

Clinical characteristics were examined based on clinical records and referral letters from other hospitals. Outcome was evaluated by findings at the last observation. Seizure outcome in surgically treated patients was evaluated by the findings just before surgical intervention. Intelligence quotient (IQ) was measured by Tanaka–Binet, WISCIII, and WAISIII, dependent on the age at examination. We used full scale IQ (FSIQ) for evaluation.

Genomic DNA was extracted from EDTA blood samples using MagNA Pure (Roche Applied Science, Tokyo) and sent as anonymous samples to a commercial laboratory that performed genome sequencing (Takara bio, Co LTD, Yokkaichi). *CTLA4* from 5′ non-coding region to 3′ non-coding region was divided into 10 regions, and each region was amplified by PCR using primers (Table A1). *PDCD1* from the promoter region to 3′ non-coding region was divided into 16 regions, and subjected to PCR amplification using primers (Table A1). The promoter region, six exons and 3′ non-coding region of *T-bet* were divided into 10 regions, and amplified by PCR using primers (Table A1). PCR reaction was performed in a final volume of 20 μl containing 2 μl of genomic DNA (10 ng/μl) by the following cycling conditions: initially 94 °C for 4 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 59 °C and 1 min at 72 °C. Thereafter, PCR products were purified with exonuclease and alkaline phosphatase, and the purified PCR products were subjected to forward and the reverse reactions using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Carlsbad, CA, USA). The reaction solution was purified by XTerminator (Applied Biosystems, Carlsbad, CA, USA). Sequencing was done using ABI3730×1 (Applied Biosystems, Carlsbad, CA, USA). Mutations and polymorphisms were detected using Phred/Phrap/PolyPhred software (CodonCode Corporation, MA, USA).

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Data of mutations and polymorphisms were compared with the data of Japanese and other populations obtained from Hap Map data (http://hapmap.ncbi.nlm.nih.gov/index.html.en). Hap Map project is a public international resource that will help researchers find genes associated with human disease. As HapMap data provide no phenotypic information about the samples from volunteers, we do not know their medical conditions. However, ages of volunteers were restricted above 20 years old, and they could understand the context of informed consent. As the age of volunteers is much older than the RS patients (6.8 ± 10.7), we suggest that the volunteers have few risks of RS. Current data from Japanese controls by HapMap may be used as tentative controls, until we will collect many new controls, to facilitate the genomic association study of RS.

For statistical analyses, chi-square test for trend and chi-square test were used. A p value less than 0.05 was considered as indicating a significant difference.

Results

Mean onset age of epilepsy in 29 patients (14 males, 15 females) was 6.8 ± 10.7 (mean $\pm$ SD) years (Table A2). The dominant hemisphere was involved in 15 patients, and non-dominant hemisphere in 14. Ten patients underwent surgical intervention and histological examination revealed typical features including microglia nodule, vasculogenesis on brain surface, endothelial proliferation, spongy degeneration and perivascular cuffing in ten patients; focal cortical dysplasia in four patients; and mesial temporal sclerosis in one patient with status epilepticus.

For *CTLA4*, the region downstream of exon 4 could not be examined by the (AT) 28 repeat at position 54947468–54947524, in spite of several modifications of sequencing conditions. Genomic sequencing revealed no disease-causative mutation, but detected two significant single nucleotide polymorphisms (SNPs) in *CTLA4* (Table 1). The rs231775 SNP in exon 1 (Thr 17 Ala) and rs231779 SNP in intron 1 showed significant positive genotypic and allelic associations with RS compared with Japanese controls. Mean age of onset was not significantly different between heterologous (A/G) and homologous (G/G) genotypes of rs231775. Frequencies of patients with EPC were not significantly different between heterologous (A/G) and homologous (G/G) genotypes of rs231775. The rs231776 (Intron 1), rs231777 (intron 1), rs231778 (intron 1), rs231780 (intron 1) and rs231721 (3'near) SNPs showed no significant association compared with Japanese controls. The rs231781 SNP (intron 3) had no significant association with RS onset compared with Asian controls. Rs231775 and rs231779 formed haplotypes in RS patients. In 25 patients (excluding two patients with deterioration after prolonged anesthesia) analyzed for cognitive outcome, mean IQ was not different between heterologous (A/G) and homologous (G/G) genotypes of rs231775 (Fig. 1). In 22 patients (excluding five patients with functional hemispherectomy and two patients with deterioration after prolonged anesthesia) analyzed for motor outcome, the proportion of patients without motor impairment was 4/16 (25%) in homologous genotype (G/G) and 1/6 (17%) in heterologous genotype (A/G) of rs231775.

Table 1 Single nucleotide polymorphisms (SNPs) in *CTLA4*, and *PDCD1*.

SNP	Subject	Genotype			Allele		HWE	Genotype	Allele frequency	
					Reference	SNP	χ^2	p-Value	p-Value	Odds ratio (95%CI)
rs231775		A/A	A/G	G/G	A	G				
(*CTLA4*, Exon 1,	RS	0	12	17	12	46	1.97	$p=0.0363$	$p=0.0137$	2.344 (1.175–4.676)
Thr17Ala)	Controls	15	55	42	85	139	0.20			
rs231779		C/C	C/T	T/T	C	T		$p=0.0467$	$p=0.0188$	2.257 (1.131–4.503)
(*CTLA4*, Intron 1)	RS	0	12	17	12	46	1.97			
	Controls	14	55	43	83	141	0.31			
rs34819629		G/G	G/A	A/A	G	A			$p=0.0195$	2.301 (1.134–4.671)
(*PDCD1*, Intron 2)	RS	4	9	9	17	27	0.41			
	Controls				49(CHB+JPT)	71(CHB+JPT)				
rs2227982		C/C	C/T	T/T	C	T		$p=0.0145$	$p=0.0114$	2.151 (1.179–3.924)
(*PDCD1*, Exon 5,	RS	4	12	13	20	38	0.21			
Ala215Val)	Controls	33	54	26	120	106	0.19			
rs10204525		G/G	G/A	A/A	G	A		$p=0.2979$	$p=0.2584$	1.57 (0.7145–3.450)
(*PDCD1*, Exon 5	RS	2	5	15	9	35	2.00			
(3'UTR))	Controls	10	45	58	65	161	0.09			

RS, Rasmussen syndrome; Controls, Japanese in Tokyo (JPT); CHB, Han Chinese in Beijing; HWE, Hardy-Weinberg Equilibrium values; p, chi square test.

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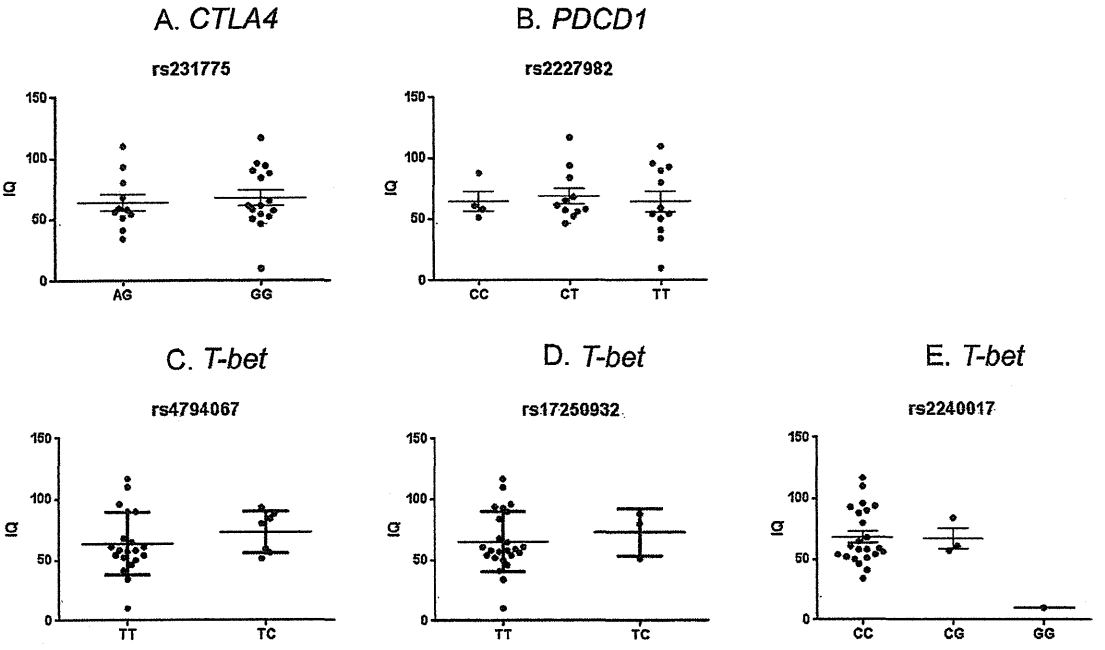

Figure 1 Cognitive outcome. *CTLA4*, cytotoxic T-lymphocyte-associated protein 4; *PDCD1*, programmed cell-death 1; *T-bet* (TBX21); IQ, intelligence quotient.

For *PDCD1*, the regions upstream of exon 1, a part of intron 1, exon 3 and exon 4 of gene could not be sequenced despite several modifications of PCR conditions. Genomic sequencing revealed no disease-causative mutation, but identified three candidate SNPs in *PDCD1* (Table 1). Rs34819629 (intron 2) showed significant allelic association compared with Asian controls [Han Chinese in Beijing (CHB) + Japanese in Tokyo (JPT)]. The rs2227982 SNP in exon 5 (Ala 215 Val) showed significant positive genotypic and allelic associations with RS compared with Japanese controls. Mean age of onset was not significantly different between patients with wild type (C/C) and those with T alleles in genotypes of rs2227982. Frequencies of patients with EPC was not significantly different between patients with wild type (C/C) and those with T alleles in genotypes of rs2227982. The rs10204525 SNP (exon 5) showed no significant genotypic and allelic associations compared with Japanese controls. The rs6705653 (intron 4) and rs2227981 (exon 5) SNPs showed significant positive genotypic association with RS compared with non-Japanese controls (Central South Africa), but no significant allelic association compared with Asian controls (CHB + JPT). The rs7419870 SNP (intron 1) showed no significant allelic association compared with Asian controls (CHB + JPT). The rs11568821 SNP (PD-1.3) was not found in RS patients. Rs6705653 and rs2227981 formed haplotypes in RS patients. In 27 patients (excluding two patients with deterioration after prolonged anesthesia) analyzed for cognitive outcome, IQ below 50 was found in 0% of wild type (C/C), 9% of heterologous (C/T), and 25% of homologous (T/T) genotype of rs2227982 (Fig. 1). In 22 patients (excluding five with functional hemispherectomy and two with deterioration after prolonged anesthesia), motor outcome was more favorable in wild-type (C/C) genotype of rs2227982 (monoplegia was the worst outcome) compared with homologous (T/T) genotype (five of whom had hemiplegia or quadriplegia).

The initial half of exon 1, exon 3 and exon 6 of *T-bet* could not be sequenced in spite of several modifications of PCR conditions. Genomic sequencing revealed no disease-causative mutation, but identified five candidate SNPs in *T-bet* (Table 2). The rs4794067 (promoter-1993), rs2240017 (exon 1, His 33 Gln) and rs2074190 (exon 1, Gly 130 Gly) SNPs showed no significant genotypic and allelic associations with RS compared with Japanese controls. The genotype data of rs2240017 in Japanese controls from Hap Map does not fit the Hardy-Weinberg equilibrium (HWE). The rs17250932 (promoter-1514) SNP showed no significant allelic associations compared with Yoruban controls. One of 26 patients had T/C genotype at NT11082994 (promoter). In 27 patients (excluding two patients with deterioration after prolonged anesthesia) analyzed for cognitive outcome, IQ below 50 was found in 19% of wild type (T/T) and 0% of heterologous (T/C) genotype of rs4794067; in 19% of wild type (T/T) and 0% of heterologous (T/C) genotype of rs17250932; and in 15% of wild type (C/C), 0% of heterologous (C/G), and 100% of homologous (G/G) genotype of rs2240017 (Fig. 1). Regarding SNPs in promoter regions (rs4794067 and rs17250932), none of the heterologous patients were quadriplegic, whereas wild-type patients (T/T) included a quadriplegic.

Discussion

Our study revealed that RS is associated with rs231775 in exon 1 of *CTLA4*, and rs2227982 in exon 5 of *PDCD1*. The rs231775 SNP in *CTLA4* involves a change of amino acid from Thr to Ala, resulting in reduced localization of CTLA4 in endoplasmic reticulum (Mäurer et al., 2002). Alteration of

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Table 2 Single nucleotide polymorphisms (SNPs) in *T-bet* gene.

SNPs	Subject	Genotype			Allele		HWE	Genotype	Allele frequency	
					Reference	SNP	χ^2	*p*-Value	*p*-Value	Odds ratio (95%CI)
rs4794067		T/T	T/C	C/C	T	C				
(Promoter-1993)	RS	23	6	0	52	6	0.39	$p=0.6127$	$p=0.561$	1.333 (0.5040–3.527)
	Controls	96	16	1	208	18	0.13			
NT11082994		G/G	G/A	A/A	G	A				
(Prompter)	RS	25	1	0	51	1	0.01			
	Controls									
rs17250932		T/T	T/C	C/C	T	C			$p=0.99$	0.9909 (0.2386–4.115)
(Promoter-1514)	RS	26	3	0	55	3	0.09			
	Controls				109 (YRI)	9 (YRI)				
rs2240017		C/C	C/G	G/G	C	G		$p=0.737$	$p=0.3552$	0.5975 (0.1987–1.797)
(Exon 1	RS	25	3	1	53	5	3.42			
His33Gln)	Controls	35	6	3	76	12	7.80			
rs2074190		A/A	A/G	G/G	A	G		$p=0.7029$	$p=0.6891$	1.238 (0.4339–3.534)
(Exonç1	RS	24	5	0	53	5	0.26			
Gly130Gly)	Controls	98	14	1	210	16	0.38			

RS, Rasmussen syndrome; Controls, Japanese in Tokyo (JPT); YRI, Yoruban in Ibadan, Nigeria; HWE, Hardy-Weinberg Equilibrium values; *p*, chi square test.

intracellular storage of CTLA4 causes reduced expression of CTLA4 on the surface of T cells, subsequently leading to peripheral tolerance dysfunction. This exon SNP (rs231775) may contribute to prolonged excretion of granzyme B from CTLs in CSF after acute infection, subsequently inducing the onset of RS. This exon SNP has been associated with several autoimmune diseases including insulin-dependent diabetes mellitus (IDDM), Graves disease, Hashimoto thyroiditis, rheumatoid arthritis, and multiple sclerosis (MS), and CTL involvement is reported in the pathogenesis of IDDM and MS (Neumann et al., 2002; Ankathatti Munegowda et al., 2011; Niland et al., 2010). Therefore, in RS also, CTLs with poor tolerance related to this exon SNP may play a pivotal role in prolonged activation of CTLs after preceding infection of RS, resulting in apoptosis of neurons by excretion of granzyme B from CTLs (Takahashi et al., 2009).

Cleavage by granzyme B is reported to play a key role in the autoantigenicity of transaldolase (TAL) and contribute to the destruction of oligodendrocytes in MS (Niland et al., 2010). Sustained cleavage of proteins by granzyme B in RS may expose cryptic epitopes to autoreactive CTLs, resulting in progression of the disease.

The rs2227982 SNP in *PDCD1* (PD-1.9) involves a change of amino acid from Ala to Val in exon 5. Exon 5 determines the cytoplasmic domain of PDCD1 (192aa-288aa), including the immunoreceptor tyrosine-based inhibitory motif (ITIM) (I/L/VXYXXL/V; 221aa-226aa) and immunoreceptor tyrosine-switch motif (ITSM) (TEYATIV; 247aa-2512aa). Ligation of the cell surface domain of PDCD1 with PD ligand leads to phosphorylation of cytoplasmic tyrosines at the ITIM and ITSM, and increases the association of src homology 2-domain containing tyrosine phosphatase 2 (SHP-2) with the ITSM in PDCD1. Recruitment of SHP-2 dephosphorylates the signaling pathway through P13K and the downstream signals through Akt, resulting in decreased production of PDCD1 and cytokines in T cells (Keir et al., 2008b). We hypothesize that the rs2227982 SNP in *PDCD1* at 215aa may affect the binding of SHP-2 to ITSM, resulting in reduced peripheral tolerance. PDCD1 deficiency in knockout mice leads to autoimmune disorders, and SNPs have been associated with several autoimmune disorders (Nishimura et al., 1999; Okazaki and Honjo, 2007). The association of rs2227982 with human diseases has been reported only in Asian populations: ankylosing spondylitis in Korean and Chinese, and type 1 diabetes mellitus in Japanese (Lee et al., 2006; Yang et al., 2011; Ni et al., 2007). These data suggest that rs2227982 is one of the risk factors for autoimmune diseases including RS in Asian populations. The fact that rs2227982 is associated with autoimmune diseases only in Asian populations suggest that the pathogenetic effect of SNP in *PDCD1* may vary depending on the genetic background, as is shown by the various effects of *PDCD1* knockout in different strains of mice (Nishimura et al., 1999; Okazaki and Honjo, 2007). More patients homologous (T/T) for rs2227982 and less wild-type patients (C/C) had poor cognitive outcome (IQ < 50). We need further studies to confirm the relationship between rs2227982 and cognitive outcome in RS.

Our analysis revealed no association between RS and SNPs of *T-bet* that may cause reduction of T-bet expression. Because T-bet is essential for the differentiation of Th0 to Th1 cells and is involved in the early induction of IFN-γ in the development of cytotoxic CD8+ effector T cells,

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reduced expression of T-bet by SNPs could inhibit the onset of autoimmune inflammatory diseases. T-bet deficiency has been shown to be protective against myasthenia gravis, inflammatory bowel diseases, multiple sclerosis, rheumatoid arthritis and type 1 diabetes mellitus (Ji et al., 2011). We speculate that intact T-bet activity without SNPs reducing Th1 differentiation do not disturb the adequate production of pro-inflammatory cytokines and CTLs, and the subsequent onset of RS.

We identified three SNPs (rs231775 and rs231779 in *CTLA4*; rs2227982 in *PDCD1*) as some of the SNPs associated with onset of Japanese RS. We need further studies in other populations to confirm these genetic associations. At the early stage of RS, patients usually manifest infrequent seizures and mild unilateral hemispheric dysfunction, so that a diagnosis of RS is difficult to establish. These genomic markers may contribute to early diagnosis of RS before the appearance of typical clinical characteristics, and early initiation of immunomodulatory treatments may result in better outcome. Furthermore, we want to conduct multivariate analysis including genetic predisposition (SNPs), age, sex, medication, etc. in larger numbers of patients and Japanese controls with clear background.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eplepsyres.2013.09.004.

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Clinical/Scientific Notes

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VITAMIN B_6-RESPONSIVE EPILEPSY DUE TO INHERITED GPI DEFICIENCY

Glycosylphosphatidylinositol (GPI) is a glycolipid that anchors many proteins to the cell surface. There are at least 26 genes involved in the biosynthesis of GPI-anchored proteins (GPI-APs).[1] Recently, many inherited GPI deficiencies (IGDs) were found using whole-exome sequencing.[2–4] The major symptoms of IGDs include mental retardation, epilepsy, coarse facial features, and multiple organ anomalies that vary in severity depending upon the degree of defect and/or position in the pathway of the affected gene. We clarified a mechanism of hyperphosphatasia, an elevated release of tissue-nonspecific alkaline phosphatase (TNAP, GPI-AP), seen in some of the patients with IGDs such as hyperphosphatasia mental retardation syndrome or Mabry syndrome caused by mutation in genes in the later stage of GPI biosynthesis.[5]

Case report. A 9-year-old boy, born to unrelated parents, presented with deafness, tetralogy of Fallot, and Hirschsprung disease; showed muscular hypotonia and facial dysmorphism; and had short fingers with hypoplasia of the distal phalanges (figure 1, A–C).

Head MRIs revealed hypomyelination and abnormal signals from the bilateral basal ganglia to the brainstem, which appear to be a cause of severe central apnea and congenital sensorineural deafness (figure 1D). At 1 year of age, he began to have intractable partial seizures. Interictal EEGs showed left hemisphere–dominant bilateral slow waves and multifocal spikes. Ictal EEGs showed rhythmic slow waves followed by apnea (figure e-1, A and B, on the *Neurology*® Web site at www.neurology.org). His developmental milestones were delayed and he never achieved speech.

Laboratory data showed elevated serum alkaline phosphatase, ranging from 1,201 to 5,959 IU/L. Serial MRIs identified progressive atrophy of the cerebellum, coupled with abnormal high-intensity lesions in the white matter over the whole brain, suggesting progressive leukoencephalopathy (figure e-2).

Supplemental data at www.neurology.org

Methods. We determined the surface levels of GPI-APs on blood granulocytes by flow cytometry. We extracted genomic DNA from the blood sample of the patient and performed target resequencing to detect mutations in 26 genes using Ion PGM Sequencer (Life Technologies, Carlsbad, CA). We obtained written informed consent from the mother of the patient and ethical approval for the study from the Osaka University Review Board.

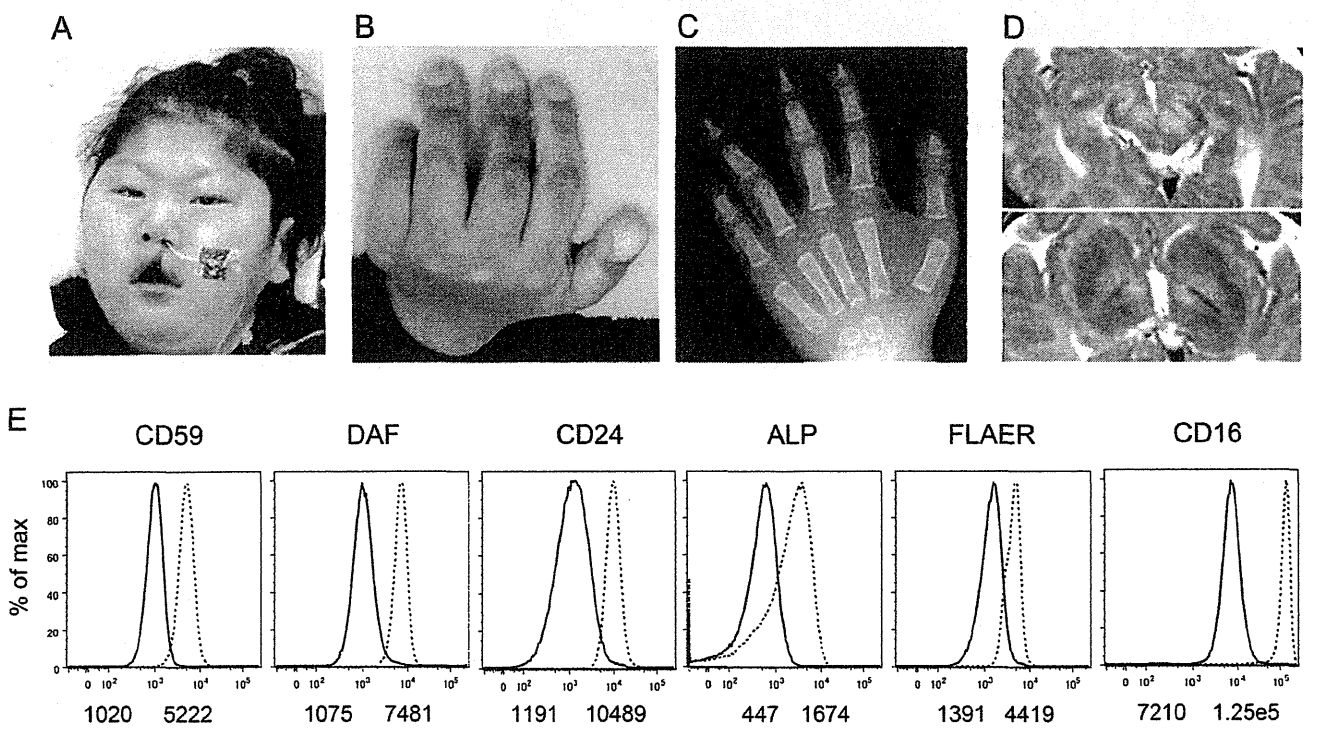
See e-Methods for more details.

Results and discussion. We found that the surface expressions of various GPI-APs on granulocytes from the patient were significantly low (7%–20% of normal levels) (figure 1E). Genomic analysis of the patient identified compound heterozygous mutations in the *PIGO* gene. Two mutations were detected, c.355C>T (p.Arg119Trp; NM_032634) and c.23497_23498del, leading to a frameshift mutation, p.Ala834fs. The father and mother were found to be heterozygous for the first and second mutations, respectively. PIGO is involved in the transfer of ethanolaminephosphate to the third mannose in GPI. We investigated the influence of these mutations on PIGO activity by transfection of *PIGO* mutant cDNAs into *PIGO*-deficient Chinese hamster ovary cells and found that p.Arg119Trp and p.Ala834fs caused severe and complete loss of PIGO activity, respectively, accounting for the decreased surface expressions of GPI-APs (figure e-3).

We treated the patient with vitamin B_6. One week after the daily oral administration of 400 mg pyridoxine (20 mg/kg), complete cessation of the seizures was observed with improvement of interictal EEGs (figure e-1C), and interruption of pyridoxine administration induced reoccurrence of habitual seizures. A previous report on a patient with Mabry syndrome showed that the seizures were controlled by pyridoxine administration, although the underlying gene was not identified.[6] Similar to our case, the patient's interictal EEG showed high-voltage slow waves and polyspike waves.[6] Overall, these data suggest that vitamin B_6 treatment may be beneficial to patients with IGD.

Patients with hypophosphatasia, caused by mutations in the *TNAP* gene, also have seizures, which have been investigated using *TNAP* knockout mice.[7] The role of TNAP on neurons is to dephosphorylate pyridoxal phosphate (PLP) to pyridoxal (PL), a membrane-permeable form, which is converted to PLP intracellularly and functions as a cofactor for glutamate decarboxylase, the enzyme that synthesizes an inhibitory neurotransmitter, γ-aminobutyric acid (GABA). *TNAP* knockout mice develop fatal seizures as a result

Figure 1 **Clinical features**



(A) Frontal facial aspect at age 9 years shows a coarse face, hypertelorism, blepharophimosis, a short nose with a broad nasal bridge, low-set ear, and cleft lip. (B, C) Clinical and radiographic hand features at age 4 years show brachytelephalangy and nail hypoplasia, especially affecting the fifth finger. (D) MRI T2 axial image at age 4 months shows high-intensity signal lesions in the bilateral basal ganglia and brainstem. (E) Surface expressions of various glycosylphosphatidylinositol (GPI)-anchored proteins (APs) on granulocytes from the patient (thick lines) were markedly decreased compared with control granulocytes (dotted lines). Shadows indicate isotype controls. The numbers indicate mean fluorescent intensities. GPI-APs were stained with fluorescence-labeled antibodies or GPI-binding aerolysin (FLAER) and analyzed by flow cytometry. ALP = alkaline phosphatase.

of severely depleted GABA levels in the brain, but can be rescued by PL treatment. As TNAP is a GPI-AP, seizures associated with IGD may be caused by a similar mechanism, a lack or a decrease of membrane-associated TNAP.

The possibility of IGD should be considered in patients with congenital seizures and mental retardation. Hyperphosphatasia is strong evidence of IGD. Flow cytometric analysis of GPI-APs on granulocytes is also useful for detection of IGD. We propose trial usage of IV or oral pyridoxine before definitive diagnosis by genomic analysis.

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Author contributions: Dr. Kuki took care of the patient and wrote the manuscript. Dr. Takahashi attended the planning of the screening system of patients with GPI anchor deficiency and served in preparation of genome DNAs. Dr. Okazaki took care of the patient and attended the planning of the screening system of patients with GPI anchor deficiency. Dr. Kawawaki took care of the patient and attended the planning of the screening system of patients with GPI anchor deficiency. Dr. Ehara took care of the patient and attended the planning of the screening system of patients with GPI anchor deficiency. Dr. Inoue attended the planning of the screening system of patients with GPI anchor deficiency, analyzed the data, and wrote the manuscript. Dr. Kinoshita attended the planning of the screening system of patients with GPI anchor deficiency, discussed the results, and wrote the manuscript. Dr. Murakami attended the planning of the screening system of patients with GPI anchor deficiency, did the experiments, analyzed the data, and wrote the manuscript.

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Immunopathological Significance of Ovarian Teratoma in Patients with Anti-N-Methyl-D-Aspartate Receptor Encephalitis

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Key Words

Anti-N-methyl-D-aspartate receptor encephalitis · Ovarian teratoma · Immunopathological study · Limbic encephalitis · Paraneoplastic syndrome

Abstract

Background: The clinical importance of ovarian teratoma in anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis has been established, however investigations of ovarian teratoma in patients with anti-NMDAR encephalitis remain limited. ***Objective:*** To clarify differences in NMDAR distribution and lymphocyte infiltration in ovarian teratoma between patients with and without anti-NMDAR encephalitis. ***Methods:*** Participants initially comprised 26 patients with ovarian teratomas. NMDAR distribution and lymphocyte infiltration in ovarian teratomas were examined using immunopathological techniques. Clinical, laboratory, and radiological data were compared between patients showing the features of encephalitis. Anti-NMDAR antibodies in the serum and cerebrospinal fluid were also measured in encephalitis patients. ***Results:*** Neuronal tissues were obtained from ovarian teratomas in 22 patients (after excluding 4 patients who did not satisfy the inclusion criteria), and the presence of NMDA receptor subunits was revealed in all patients. Lymphocyte infiltration was more frequent in the encephalitis group (n = 3) than in the non-encephalitis group. In particular, dense B-lymphocyte infiltration near neural tissues was observed in the encephalitis group. ***Conclusions:*** Differences in lymphocyte infiltration in ovarian teratomas between anti-NMDAR encephalitis and non-encephalitis patients suggest the immunological importance of the ovarian teratoma as the site of antigen presentation in anti-NMDAR encephalitis.

In recent years, anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis has been receiving attention due to its clinical characteristics such as female predominance, limbic encephalitis-like clinical features, and the presence of an ovarian teratoma in over 50% of cases [1, 2]. Interestingly, this encephalitis has been associated with the antibody (Ab) against NMDAR subtypes 1 and 2B (NR1/NR2) heteromers of NMDAR in the serum and cerebrospinal fluid (CSF) [1, 3]. Several clinical features, such as responsiveness to immunotherapy and surgical teratoma removal and the presence of anti-NMDAR Abs in the serum and CSF, strongly suggest that autoimmunity may be central to the pathogenesis of anti-NMDAR encephalitis [4–6]. Although the relationship between anti-NMDAR encephalitis and ovarian teratomas has been considered, histological investigations of ovarian

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