SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article at http://dx.doi.org/10.1016/j.cmet.2015.01.019.

AUTHOR CONTRIBUTIONS

F.-Y.W. and K.T. designed the experiments and wrote the manuscript. F.-Y.W. and B.Z. performed the experiments. Takeo Suzuki and Tsutomu Suzuki performed the mass spectrometry experiments. H.H., K.M., and Y.O. performed cardiac examinations. Y.U. and S.M. performed TAC surgery and cardiac examinations. H. Michiue, A.F., and H. Matsui performed electron microscopy. Y.K. provided the blood samples. N.T., P.X., and T.K. performed the qPCR-based examination of tRNA modifications.

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Immediate suppression of seizure clusters by corticosteroids in PCDH19 female epilepsy

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ABSTRACT

Purpose: The pathomechanism and treatment of *PCDH19* female epilepsy (*PCDH19*-FE) remain unclear. Here, we report that corticosteroids are effective for control of the seizure clusters or other acute symptoms of *PCDH19*-FE and argue for the possible involvement of a compromised blood-brain barrier (BBB) in its pathogenesis.

Methods: The efficacy of corticosteroids was retrospectively reviewed in five Japanese patients with *PCDH19*-FE. The results of antibody assays against the *N*-methyl-D-aspartate-type glutamate receptor (abs-NR) in serum/cerebrospinal fluid were also compiled.

Results: Corticosteroid treatments significantly improved the acute symptoms, including seizure clusters, in all cases, most often immediately after the initial administration. However, the effect was transient, and some seizures recurred within a few weeks, especially in association with fever. Serum and/or cerebrospinal fluid abs-NR were detected in all patients. Target sequences of the detected antibodies were multiple, and the titers tended to decrease over time. In one patient, immunohistochemical analysis using rat hippocampal slices also revealed serum antibodies targeting an unknown epitope in neuronal cytoplasm.

Conclusion: Our findings imply an involvement of inflammatory processes in the pathogenesis of *PCDH19*-FE and therapeutic utility for corticosteroids as an adjunctive option in acute treatment. *PCDH19* is well expressed in brain microvascular endothelial cells and thus its impairment may cause BBB vulnerability, which may be ameliorated by corticosteroids. The abs-NR detected in our patients may not indicate an autoimmune pathomechanism, but may rather represent non-specific sensitization to degraded neuronal components entering the general circulation, the latter process facilitated by the BBB vulnerability.

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1. Introduction

A heterozygous defect in the gene encoding protocadherin 19 (*PCDH19*) causes early-onset intractable epilepsy in females (*i.e.*, *PCDH19*-related female epilepsy, *PCDH19*-FE, or previously, epilepsy and mental retardation limited to females, EFMR) [1]. PCHD19, an adhesion molecule of the δ2-protocadherin subclass of the cadherin superfamily, is highly expressed in the vertebrate brain. δ-Protocadherins are intimately involved in brain development and neural functions, as well as in many neurological diseases [2]. However, the homophilic adhesion capacity of PCDH19 by itself is low, and its exact function remains unclear.

The hallmark clinical feature of *PCDH19*-FE is recurrent seizure clusters consisting of brief focal seizures and/or generalized convulsions, which can be triggered by febrile or afebrile illnesses [3]. The seizures do not recur regularly, but once they recur, the cluster continues for days to weeks despite multiple treatments. Conventional antiepileptic drugs fail to control or prevent most of these seizures. Ictal symptoms and EEG findings indicate that the seizures mainly involve the limbic system and medial frontal region [4].

The clinical features indicate a possible immune/inflammation involvement in seizure generation, which could be a non-genetic modifier of the disease phenotype. In agreement with this, we have previously reported patients showing excellent efficacy of corticosteroids for seizure clusters [5]. We have also encountered cases having antibodies to the *N*-methyl-D-aspartate (NMDA)-type glutamate receptor (abs-NR) in the serum or cerebrospinal fluid (CSF). Abs-NR cause limbic encephalitis, predominantly in young women (anti-NMDA receptor encephalitis) [6], but may also appear secondarily and non-specifically in various neurologic diseases including epilepsy [7]. In the latter case, abs-NR are not significantly involved in the disease pathogenesis.

This study aims to explore whether corticosteroids have an ability to improve the seizures in *PCDH19*-FE and if any immune mechanism is involved. We retrospectively reviewed and summarized the clinical results of corticosteroid treatments as well as the results of an assay for abs-NR in Japanese patients. The potential significance of these findings with regard to the pathomechanisms of this disorder is also discussed.

2. Methods

2.1. Patients

Japanese patients with *PCDH19*-FE who received corticosteroid treatments and/or underwent the ab-NR assay were retrospectively studied. They were genetically diagnosed at Fukuoka University[5] and clinical details were collected from their doctors in charge. Since the patients were children, the doctors obtained written informed consent from the parents before the blood and/or CSF samples were drawn for genetic analysis of *PCDH19* and/or for assay for ab-NR. Genetic analysis of *PCDH19* was approved by the ethics committee of Fukuoka University. The ab-NR assay was approved by the National Epilepsy Center, Shizuoka Institute of Epilepsy and Neurological Disorders.

2.2. ab-NR assay

The ab-NR assay was performed at National Epilepsy Center, Shizuoka Institute of Epilepsy and Neurological Disorders. Experimental details have been described elsewhere [8]. Briefly, serum and CSF samples were examined by enzyme-linked immunosorbent assay (ELISA) or, in one patient, by immunoblot analysis targeting GluN2B. The ELISA target peptides were the extracellular N-terminal (NT) and/or intracellular C-terminal (CT) regions of the GluN2B, GluN1, and GluD2 subunits (Supplementary Table 1). Titers were determined by comparing the optical densities of our patients with those of 35 patients with non-inflammatory focal epilepsy, who served as controls. The results were expressed as number of standard deviations of the controls (SD) from the mean of the controls and were considered positive when ≥2 SD.

2.3. Immunohistochemical analysis for anti-neuronal autoantibodies

In two patients, the presence of anti-neuronal autoantibodies was further examined immunohistochemically using rat hippocampal slices exposed to serum or CSF from the patients. Experimental details are described in Supplementary information.

3. Results

Five patients (Patients 1–5) received corticosteroid treatments mainly during the acute phase before and/or after the diagnosis of *PCDH19*-FE. Abs-NR were examined in these patients mostly before corticosteroid administration and in four other patients (Patients 6–9). These tests were performed because autoimmune or inflammatory processes were clinically suspected in the pathogenesis of the epilepsy, although none of these patients except Patient 4 showed pleocytosis or an elevation of CSF protein level. Outlines of Patients 1, 2, and 5–9 have been described previously [3,5].

3.1. Efficacy of corticosteroid treatments

Treatment details and results are summarized in Table 1. The details of the clinical courses are described in Supplementary information. Overall, corticosteroids dramatically improved acute neurological symptoms: ongoing seizure clusters in Patients 1–3 and 5, and an acute encephalopathic episode that developed after a seizure cluster in Patient 4, were controlled. In most cases, the improvement was achieved after the first administration. In the cases of seizure cluster, the initial administration was conducted well in advance of the expected time of spontaneous remission of the cluster. Furthermore, in Patient 3, corticosteroids were initiated on the second day of each cluster to confirm that the cluster continued for more than 1 day despite midazolam administration. Regarding drugs and dosages, four young patients (Patients 1–4) received an intravenous drip infusion of 10–30 mg/kg methylprednisolone once daily, for up to 3 days. Patient 5 received an intravenous infusion of 0.35 mg/kg prednisolone once or twice, depending on the cluster, followed by oral administration of prednisolone at 1 mg/(kg·day) at age 11. However, as observed in Patients 1, 3, and 5, the effect was fundamentally transient; seizure clusters often recurred within a few weeks, especially when fever appeared.

For Patient 4, methylprednisolone was used at age 1 for an encephalopathic episode with decline of consciousness and systemic weakness, which abruptly developed 3 days after the termination of a one-day seizure cluster. EEG showed an increase of δ-waves, but no ictal activity. Mild CSF pleocytosis (85 cells/μL) was identified a week before the episode. These symptoms completely disappeared immediately after the initial administration of methylprednisolone.

For Patient 1, corticosteroids were administered prophylactically after age 3, with 3 days of oral betamethasone or prednisolone administration at times of fever appearance. After starting this treatment, no or only mild recurrences (not requiring hospitalization) were observed, even during fever.

Table 1
Details and efficacy of corticosteroid therapy.

Pt no	*PCDH19* mutation	Age at onset (m)	Age at CS TX	CS	Route & dose	Target symptom	Simultaneous TX	Usual duration of Sz cluster	Result	Present intellect
1	p.L719*	13	2y4m	mPSL	IV, 30 mg/kg, 3d	Sz cluster	MDL CBZ CZP VPA LTG LEV	Days ~2 wk	Disappeared after 1st IV	Normal 5y1m
			2y10m	mPSL	IV, 30 mg/kg, 3d	Sz cluster			Disappeared after 1st IV Recurred in 2 wk w/fever[a]	
			2y11m	mPSL	IV, 30 mg/kg, 3d	Sz cluster			Disappeared after 1st IV Recurred in 1wk w/fever	
			3y0m	mPSL	IV, 10 mg/kg, 3d	Sz cluster			Disappeared after 1st IV	
			3y4m	BET	Oral, 0.01 mg/kg, 3d When fever appeared	Sz prevention			No or mild recurrence	
			4y1m	PSL	Oral, 1–1.5 mg/kg, 3d When fever appeared	Sz prevention			No or mild recurrence	
2	p.K120Rfs*3	10	10m	mPSL	IV, 30 mg/kg, 3d	Sz cluster	MDL PB ACV IVIG EDV	–	Disappeared after 1st IV Recurred in 1wk	Moderate delay 3y
3	p.D417H p.D596Y	5	1y11m	mPSL	IV, 20 mg/kg, 2d	Sz cluster	MDL fPHT CLB LEV KBr DZP	Days ~2wk	Disappeared after 1st IV	Normal 2y8m
			2y1m	mPSL	IV, 20 mg/kg, 3d	Sz cluster			Disappeared after 1st IV	
			2y2m	mPSL	IV, 20 mg/kg, 2d	Sz cluster			Disappeared after 1st IV	
			2y5m	mPSL	IV, 10 mg/kg, 1d *fol. by* 20 mg/kg, 1d	Sz cluster			Disappeared after 2nd IV	
			2y7m	mPSL	IV, 20 mg/kg, 1d	Sz cluster			Disappeared after 1st IV Recurred in 9d w/flu	
			2y7m	mPSL	IV, 20 mg/kg, 2d	Sz cluster			Disappeared after 1st IV	
4	p.D596G	6	1y0m	mPSL	IV, 30 mg/kg, 3d	Encephalopatic symptoms	CBZ fPHT LDC PB	1d	Disappeared after 1st IV	Hyperactive 1y6m
5	p.D45Gfs*43	8	11y5m	PSL	IV, 0.35 mg/kg x1 *fol. by* Oral, 1 mg/kg[b]	Sz cluster	KBr CZP	Half a day	Disappeared after 1st IV	Moderate delay 11y8m
			11y6m	PSL	IV, 0.35 mg/kg x1 *fol. by* Oral, 1 mg/kg	Sz cluster			Disappeared after 1st IV Recurred in 1wk w/fever	
			11y6m	PSL	IV, 0.35 mg/kg x1 *fol. by* Oral, 1 mg/kg	Sz cluster			Disappeared after 1st IV	
			11y8m	PSL	IV, 0.35 mg/kg x2 *fol. by* Oral, 1 mg/kg	Sz cluster			Disappeared after 2nd IV	

[a] Noted when seizures recurred within 3 weeks after corticosteroid administration.
[b] In Patient 5, oral prednisolone was gradually tapered off. Pt no, Patient number; m, month(s); CS, corticosteroid; TX, treatment; Sz, seizure; y, year(s); mPSL, methylprednisolone; BET, betamethasone; PSL, prednisolone; IV, intravenous route; d, day(s); MDL, midazolam; CBZ, carbamazepine; CZP, clonazepam; VPA, valproic acid; LTG, lamotrigine; LEV, levetiracetam; wk, week(s); PB, phenobarbital; ACV, acyclovir; IVIG, intravenous immunoglobulin; EDV, edaravone; *fol. by*, followed by; fPHT, fosphenytoin; CLB, clobazam; KBr, potassium bromide; DZP, diazepam; LDC, lidocaine.

3.2. *ab-NR and further anti-neuronal autoantibody assays*

Eight of the nine patients who underwent the assay showed positivity to multiple epitopes in the serum or CSF (Patients 1–7 and 9, 88.9%, Table 2). The epitopes included GluN1-NT, which has been reported to be critical for the emergence of neuropsychiatric symptoms in anti-NMDA-receptor encephalitis [6]. Patients 1 and 2 had high CSF titers of antibodies during the acute phase (>10 SD). Patient 6 showed positivity in the CSF at onset and in serum half a year later. Patients 3, 5, and 9 underwent follow-up assays, and their titers were found to decrease over time. In Patient 5, immunohistochemical analysis during seizure recurrence at age 11, using serum drawn before prednisolone administration, revealed autoantibodies to the cytoplasm of hippocampal neurons, as demonstrated in hippocampal slices taken from rats (Supplementary Fig. b). The assay failed to identify the epitope. These results suggest that following seizure clusters, an immune reaction occurs non-specifically to degraded neuronal proteins, including NMDA-type glutamate receptor, inside and subsequently outside the brain. Such reactions appear to be strong at early ages, but do not show a uniform pattern.

4. Discussion

This study revealed the therapeutic potency of corticosteroids for acute symptoms in *PCDH19*-FE. The rapid and efficient response was remarkable and might be a useful indicator for this disease. The cases of Patients 1 and 9 suggested that oral corticosteroids taken during interictal periods might exert some prophylactic effects, but further assessments are necessary to establish this.

Table 2
Results of assay for antibodies to *N*-methyl-D-aspartate-type glutamate receptor.

Pt no	*PCDH19* mutation	Age at onset (m)	Age at assay	Serum					Cerebrospinal fluid				
				GluN2B		GluN1	GluD2		GluN2B		GluN1	GluD2	
				NT	CT	NT	NT	CT	NT	CT	NT	NT	CT
1	p.L719*	13	2y5m	1.61	**3.12**		1.93	0.45	**14.10**	**15.10**		**10.80**	**8.10**
2	p.K120Rfs*3	10	11m						**15.42**				
			4y2m	**2.21**	1.53	**3.58**	**3.63**						
3	p.D417H	5	11m	**2.17**	**2.94**	**3.62**	**2.85**						
	p.D596Y		1y0m						**3.39**	**3.28**	**2.81**	**2.35**	
	Heterozygous		1y7m	**2.65**	**2.21**	**2.17**	1.95						
			1y10m	1.23	0.37	0.72	0.99						
4	p.D596G	6	1y0m						**2.67**	**2.39**	1.51	1.68	
5	p.D45Gfs*43	8	9m	+					–	+			
			3y5m	–					–				
6	Whole del	10	11m	0.57	1.70		0.46	(0.34)	1.61	**2.73**		1.34	0.75
			1y5m	**2.33**	**2.73**		**2.48**	0.92	0.46	1.18		1.13	0.70
7	p.Asn340Ser	9	8y1m	**3.49**	**2.07**	**9.55**	1.87						
8	p.R198L	7	6y5m	1.70	0.90	1.78	1.68						
9	p.Y366Lfs*10	5	6y0m	**9.52**		**12.50**	**9.20**		−0.88		−1.37	−1.08	
			8y5m	0.60	(0.35)	0.25	0.37						

Results are expressed as standard deviation (SD, enzyme-linked immunosorbent assay) or plus/minus sign (immunoblot, Patient 5 only). Bold values denote positivity (≥2 SD or +). Blanks are unexamined. *Patients who received corticosteroid treatments. Pt no, Patient number; m, month(s); NT, N-terminal; CT, C-terminal; y, year(s); Whole del, Whole *PCDH19* deletion.

Despite such efficacy, short-term administration of corticosteroids provided only transient effects and had no potential for preventing further seizure clusters. Therefore, the clinical benefit will be limited for cases with frequent recurrences such as Patients 3 and 5. Moreover, excessive amounts of corticosteroids may have adverse effects, actually increasing seizure-proneness as described below. These findings suggest that the attending physician should consider discontinuing corticosteroid administration for acute treatment soon after seizure disappearance. Indication for treatment should be based on various patient conditions such as age, seizure severity, and comorbid infections.

Corticosteroids may exert primarily excitatory/pro-convulsive actions on brain neurons both *in vitro* and *in vivo*, especially under chronic stress conditions such as epilepsy [9]. Despite this, the therapeutic efficacy of corticosteroids is well established in many intractable epilepsies such as West syndrome, Landau-Kleffner syndrome, and autoimmune epilepsy/encephalitis [10]. In these diseases, clinical improvements after corticosteroid administration are usually delayed and are probably mediated by mechanisms such as immunosuppression and feedback inhibition of corticotropin-releasing hormone secretion. In our patients, however, the clinical effects appeared quickly, usually immediately after the initial administration. Moreover, significant brain inflammation was not found, suggesting a particular pathogenesis and mechanism of action of corticosteroids in *PCDH19*-FE.

Such a mechanism may be restoration of blood-brain barrier (BBB) integrity [11]. This is known to be a crucial action of corticosteroids, the underlying molecular basis of which has been partially elucidated [12]. Seizures are easily triggered by a mild breakdown of brain homeostasis due to a compromised BBB, which can be prevented or alleviated by corticosteroid administration [13]. Although PCDH19 is abundantly expressed in brain neurons, it is unclear how the heterozygous mutation in *PCDH19* leads to epilepsy in females. "Cellular interference," that is, the presence of somatic mosaicism in PCDH19 expression between normal and abnormal neurons, is the currently proposed explanation for such sex-specific pathogenesis, but the concept is very theoretical and has yet to be established. However, *PCDH19* is also expressed in the BBB. In mouse, the BBB-specific transcriptome included *PCDH19*, and *PCDH19* expression in microvascular endothelial cells was significantly higher in the brain than in the liver or lung [14]. Although currently no human evidence exists, this study suggests that PCDH19 plays a role in the BBB, and speculatively, that *PCDH19* mutation leads to a functional BBB vulnerability, that underlies the pathogenesis of *PCDH19*-FE.

Interestingly, *PCDH19* expression may be significantly altered in the BBB during systemic inflammation, which is the predisposing factor for seizure recurrence in *PCDH19*-FE. In cultured mouse brain microvascular endothelial cells, treatment with lupus serum or an activated complement, C5a, significantly down-regulated miR-320a expression [15]. *PCDH19* is one potential target of this microRNA. Although systemic inflammation will impair BBB function to some extent [16], the impairment may be exacerbated in patients with *PCDH19*-FE, probably due to PCDH19 insufficiency. The resulting seizure clusters will further exacerbate the BBB dysfunction by inducing brain inflammation. Corticosteroids may ameliorate such dysfunction and thus the acute neurological symptoms.

Other aspects of *PCDH19*-FE and the findings of this study are also consistent with the BBB hypothesis: The seizures mainly involve the limbic system, which is anatomically close to some of the periventricular regions that lack a BBB due to their endocrine roles; seizure occurrence and remission are strongly age-related, and BBB integrity also develops age-dependently; abs-NR in our patients covered multiple epitopes of various subunits, and Patient 8 had antibodies to some neuronal cytoplasmic component(s) as well. These results indicate that anti-neuronal antibodies may be produced in *PCDH19*-FE non-specifically and commonly. Various neuronal proteins will be degraded by recurrent seizures and the compromised BBB may then facilitate leakage of such degraded proteins into the bloodstream. This may induce non-specific sensitization to them outside the brain, resulting in the high rate of anti-neuronal autoantibody positivity seen in our patients. Thus, the abs-NR found in our patients does not represent an autoimmune pathogenesis.

However, a possible modifying effect of the abs-NR on the neurological phenotype in our patients with *PCDH19*-FE can also be considered. The seroprevalence of abs-NR has been found to be identical between patients with psychiatric diseases, including schizophrenia, and healthy individuals, but the disease phenotypes were more severe in patients with compromised BBBs than in those without [17]. Serum abs-NR could have passed the compromised BBB and worsened the psychiatric symptoms of these patients. In anti-NMDA-receptor encephalitis, a primary involvement of antibodies to GluN1-NT in causing its neuropsychiatric symptoms has been suggested [6]. Therefore if BBB compromise exists in *PCDH19*-FE, the anti-GluN1-NT could be partially responsible for the neuropsychiatric symptoms also seen in this disease.

This study proposes corticosteroid treatment as an efficacious adjunctive treatment for the acute symptoms of *PCDH19*-FE and suggests BBB involvement in this disease. Although *PCDH19*-FE is rare, future multicenter clinical trials should be conducted to verify the acute and long-term efficacy of corticosteroid treatment and to define the therapeutic indications of such treatment, since the present study is only a retrospective review of 5 patients. In addition, reliable animal models should be devised to elucidate the entire molecular pathogenesis of *PCDH19*-FE. Importantly, not only must neurons be studied, but also other actors such as the BBB and the inflammatory system as well.

Conflict of interest

All authors wish to confirm that there are no known conflict of interest associated with this publication.

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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.seizure.2015.02.006.

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