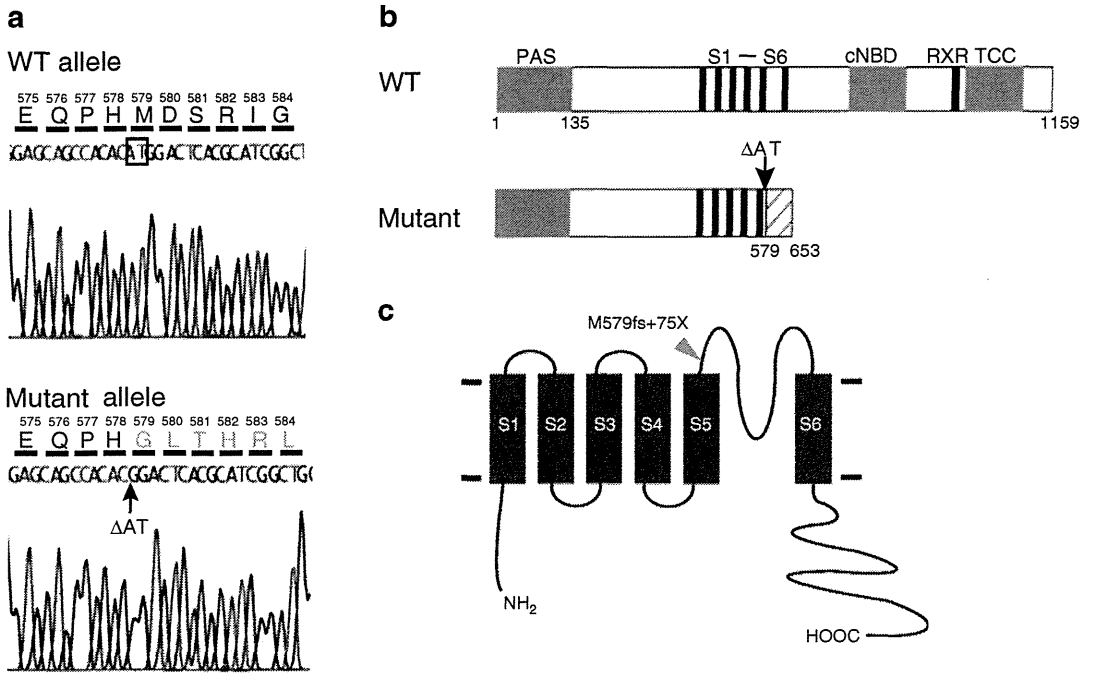

Fig. 2 Sequence analysis of exon 7 in *hERG*. **a** Direct sequencing revealed a 2-bp deletion of nucleotides 1735–1736 in exon 7 of *hERG*. The putative amino acid sequence is shown above the nucleotide sequence. **b** Schematic diagram of the *hERG* subunit, showing the location of the hERG truncation mutation and domains or regions proposed to be involved in hERG channel assembly and trafficking. PAS: Per, Arnt, and Sim domains; S1–S6: transmembrane domain; S1–S4: voltage sensor domain; S5–S6: K^+ selective pore; cNBD: cyclic nucleotide binding domain (residues 750–870); RXR: ER retention motif (residues 1005–1007); TCC: tetramerizing coiled-coil domain (residues 1018–1122). **c** Schematic representation of the predicted topology of the wild-type *hERG* potassium channel and the location of the deletion (*orange triangle*)

Analysis of mRNA isolated from whole blood

To determine the pathogenic effects of *hERG*(ΔAT), we used RT-PCR to analyze the *hERG* transcripts in the subject. We prepared RNA from whole blood samples from both the patient and the control subject. cDNA synthesized from DNase I-treated RNA was used as a template for PCR amplification (Fig. 3a). The RT-PCR products of *hERG* transcripts in the *hERG*(ΔAT) subject and the control both showed an approximately 370-bp band by electrophoresis (Fig. 3b). Direct sequencing of the RT-PCR products of the patient revealed the presence of transcripts from both wild-type (*hERG*(WT)) and mutant (*hERG*(ΔAT)) alleles. However, in the chromatograms, the sequence corresponding to the *hERG*(ΔAT) allele had lower peaks (Fig. 3c), suggesting that fewer transcripts were derived from the *hERG*(ΔAT) allele than from the *hERG*(WT) allele. To rule out the possibility of allelic variation in *hERG* mRNA expression, we compared the levels of transcripts derived from the 1692A and 1692G alleles of the control. The results indicated almost equal levels of transcripts from these two alleles, suggesting a lack of allelic variation in *hERG* mRNA expression of common alleles.

Expression of hERG(WT) and hERG(ΔAT) channel subunits in HEK-293 T cells

We used Western blot analysis to assess the expression of wild-type or mutant channel subunits in HEK-293 T cells that were transfected with *hERG*(WT) and *hERG*(ΔAT) cDNA. The anti-hERG antibody recognized bands corresponding to proteins larger than ~135 kDa in *hERG*(WT)-transfected cells and a protein of ~76 kDa in *hERG*(ΔAT)-transfected cells (Fig. 4). The sizes of the main proteins detected for the hERG(WT) and hERG(ΔAT) subunits are consistent with that reported for the wild-type subunit [17] and the expected size of the truncated protein. The amount of hERG(WT) protein detected was at least 50 times that of the hERG(ΔAT) protein (Fig. 4). In addition, the ratio of the protein expression levels of the hERG(WT) and hERG(ΔAT) subunits in HEK-293 T cells that were co-transfected with *hERG*(WT) and *hERG*(ΔAT) cDNAs was similar to the ratio of the protein levels in singly transfected cells. These results suggest that the hERG(ΔAT) subunit protein was expressed at a lower level than that of the hERG(WT) subunit and that the expression of wild-type and mutant channel subunits did not interfere with each other.

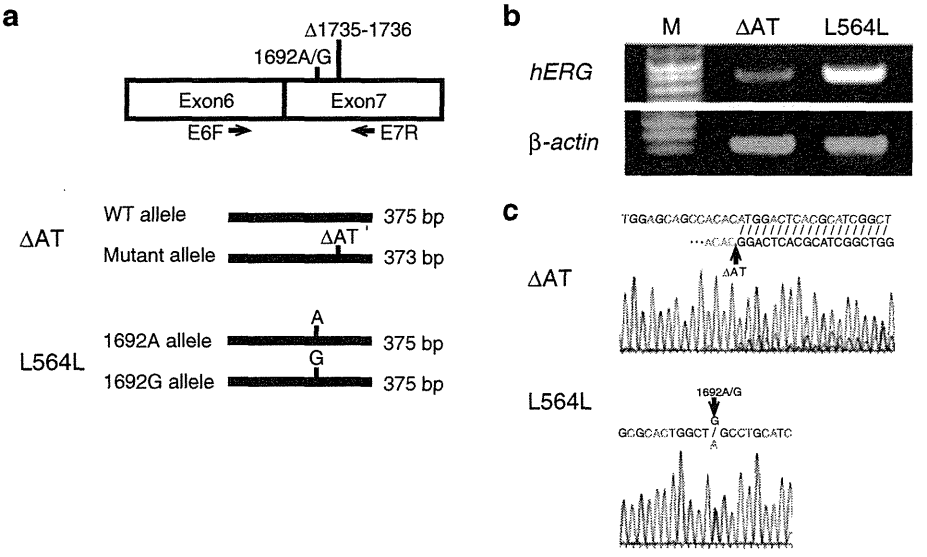

Fig. 3 Expression of *hERG* in blood cells. **a** Schematic diagram showing the position of the mutation and the sizes of the nucleotide fragments from alleles of the *hERG*(ΔAT) carrier, (ΔAT), and the 1692A/G carrier, (L564L). Forward primer E6F in exon 6 and reverse primer E7R in exon 7 were used in the RT-PCR analysis. **b** Using RT-PCR analysis, the expression levels of *hERG* and *β-actin* were determined in whole blood samples from both the *hERG*(ΔAT) and the 1692A/G carrier. **c** Direct sequencing of the gel-extracted RT-PCR products demonstrated that they were derived from both *hERG*(WT) (major) and *hERG*(ΔAT) (minor) transcripts. In contrast, the PCR products derived from the 1692A/G alleles (hERG(L564L)) arose from equal amounts of transcripts from both alleles

Subcellular localization and expression of hERG(WT) and hERG(ΔAT) channel subunits

To reveal the heterologous expression of hERG(WT) and hERG(ΔAT) channel subunits in HEK-293 T cells, we constructed C-terminally HA-tagged *hERG*(WT) and Myc-tagged *hERG*(ΔAT) expression vectors. We then co-transfected these tagged expression vectors into HEK-293 T cells and used confocal microscopy and anti-HA and anti-Myc antibodies to examine the subcellular localization and expression of these subunits. We detected cells

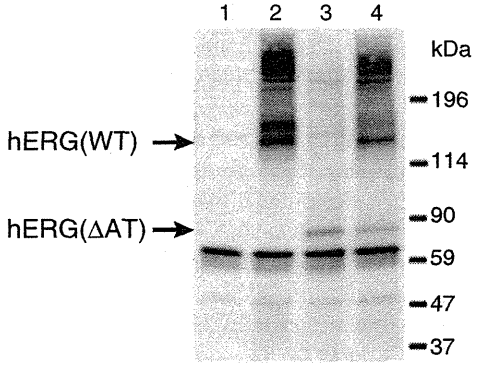

Fig. 4 Western blot analysis of hERG(WT) and hERG(ΔAT). Extracts of HEK-293 T cells were transfected with pCAGGS vector (*lane 1*), *hERG*(WT) expression vector (*lane 2*), *hERG*(ΔAT) expression vector (*lane 3*), and both *hERG*(WT) and *hERG*(ΔAT) (*lane 4*) underwent Western blot analysis using an anti-hERG antibody. The *arrows on the left* indicate the locations of the hERG(WT) and hERG(ΔAT) proteins; the positions of protein size markers are indicated on the *right side*

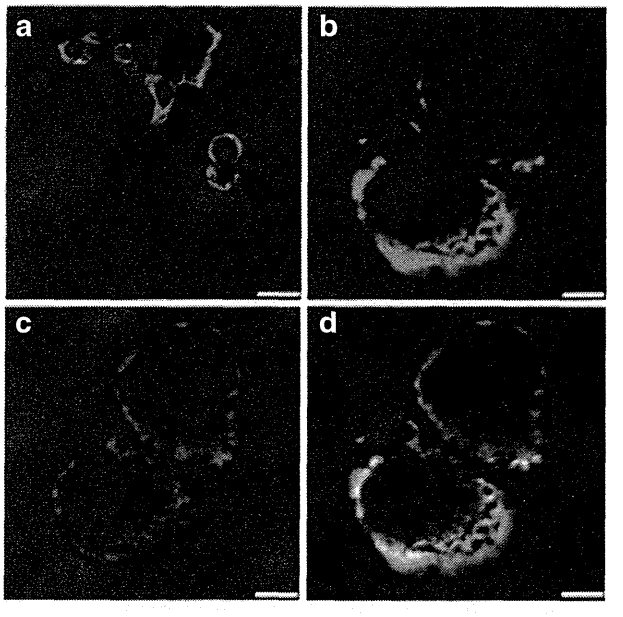

Fig. 5 Immunolabeling of HEK-293 T cells transiently transfected with HA-tagged *hERG*(WT) and Myc-tagged *hERG*(ΔAT). The immunofluorescence signals due to anti-HA antibody (*green* in **a**, **b**, and **d**) and anti-Myc antibody (*magenta* in **a**, **c**, and **d**) were merged with that from 4′,6-diamidino-2-phenylindole (*blue* in **a** and **d**). Scale bars= 25 μm (**a**) and 5 μm (**b**–**d**)

expressing HA-associated signals easily, but cells expressing Myc-associated signals were sparse (Fig. 5a). Most of the HA- and Myc-associated signals were segregated subcellularly, with those of HA-tagged hERG(WT) at the periphery of cells, whereas those of Myc-tagged hERG(ΔAT) remained intracellularly (Fig. 5b, c, d). These results suggest that the Myc-tagged hERG(ΔAT) subunit was expressed at lower levels compared with the HA-tagged hERG(WT) subunit and that what little mutant protein was produced remained localized within the cells.

Electrophysiological analysis of the hERG(ΔAT) subunit

To examine whether the hERG(ΔAT) subunit can form functional channels in the absence or presence of the hERG(WT) subunit, we compared whole cell currents from HEK-293 T cells transfected with hERG(ΔAT) cDNA, both with and without hERG(WT) cDNA. The transfected cells were stimulated using a double-step voltage protocol (Fig. 6a, schematic). In the first step (i.e., at a relatively high voltage), the hERG channel underwent activation as well as inactivation. During the second step (i.e., at a less positive voltage), the hERG channel was relieved from inactivation and produced a large "tail" current (Fig. 6a, hERG(WT) traces). The tail current measured under these conditions included virtually no component mediated by the HEK-293 T cell's native voltage-dependent current [18] because its amplitude did not differ notably between cells transfected with *hERG*(WT) cDNA and those transfected with *GFP* cDNA alone (Fig. 6b).

We presume that *hERG*(WT)-transfected cells displayed hERG channel-mediated tail current, given that its peak density at a first-step voltage of 60 mV (49.1±5.4 pA/pF [mean± SEM, n=18]) was significantly (P<0.001, rank-sum test) larger than that of *GFP*-transfected cells (3.0±0.6 pA/pF; n=12; Fig. 6b). By contrast, the *hERG*(ΔAT)-transfected cells did not display similar hERG channel-mediated tail currents because their peak density (3.7±0.6 pA/pF; n=14) did not differ from that of the *GFP*-transfected cells (Fig. 6b). In addition, the peak density of the tail current of the *hERG*(ΔAT)-transfected cells was significantly (P<0.001, rank-sum test) smaller than that of the *hERG*(WT)-transfected cells. These results suggest that the hERG(ΔAT) subunits cannot form functional channels on their own.

The amplitude of the tail current produced by the HEK-293 T cells transfected with a 1:1 mixture of *hERG*(ΔAT) and *hERG*(WT) cDNAs (peak current density at a first-step voltage of 60 mV, 50.4±7.9 pA/pF; n=14; Fig. 6b) was comparable to that of the *hERG*(WT)-transfected cells. One possibility suggested by this result is that hERG(ΔAT) subunits might form functional channels with the aid of hERG(WT) subunits. Alternatively, homomeric hERG(WT) channels might account for a predominant portion of the tail current, with the hERG(ΔAT) subunit-containing channels making only slight or no contribution to the tail current. To assess these possibilities, we measured the tail current in cells transfected with a 30:1 mixture of *hERG*(ΔAT) and *hERG*(WT) cDNAs, in which the mutant subunits outnumbered the wild-type subunits. In this case, the peak current density (24.9±6.9 pA/pF, n=10) was significantly (P<0.001, rank-sum test) less than that of cells transfected with *hERG*(WT) only (Fig. 6b).

To quantify the voltage dependence of the activation extent, the Boltzmann equation was fitted to the plot of the normalized peak tail current amplitude against first-step voltage (Fig. 6c). The half-maximal activation voltage (V_{half}) and slope (K) of the cells transfected with the 30:1 mutant/WT mixture (−3.28±3.53 mV and 8.99±1.19, respectively; n=10) were not significantly different from those of *hERG*(WT)-transfected cells (−0.30±1.59 mV and 8.18±0.79, respectively; n=18). In addition, the activation time-course depicted by plotting the peak tail current amplitude against the varied duration of the first-step voltage did not differ notably between the *hERG*(WT)-transfected cells (time constant 125.0±14.1 ms; n=17) and the 30:1-transfected cells (121.7±15.5 ms; n=10; Fig. 6d). These results suggest that the hERG(ΔAT) subunit does not influence the kinetic properties of the hERG(WT) channels.

Discussion

Since many arrhythmia patients present genetic anomalies, cases of sudden unexpected deaths can be expected where there are family histories of arrhythmias. Thus, molecular biological analyses would be useful for exploring the cause of such cases. Of course, careful ethics considerations are essential. Our examination of the present case demonstrated that the patient's cause of death was ventricular arrhythmia due to an unreported ion channel disorder. Such analyses would be useful for not only exploring the cause of death, but also in contributing to clinical and basic research on arrhythmia.

This study investigated the molecular and electrophysiological properties of the product of a novel ΔAT mutant *hERG* we identified from a patient who died from SCD. This mutation leads to the frameshift M579fs+75X and the production of a truncated hERG subunit that lacks the C-terminus. Although the patient was heterozygous for the ΔAT mutation, she displayed mild symptoms of QTc prolongation on ECG, dilatation of the left ventricle at autopsy, and histologically apparent interstitial fibrosis of the left ventricle and atrioventricular conduction system.

More than 30 % of known *hERG* mutations are of the nonsense or frameshift type that introduce premature termination codons [19, 20]. mRNAs containing a premature termination codon more than 50–55 nucleotides upstream

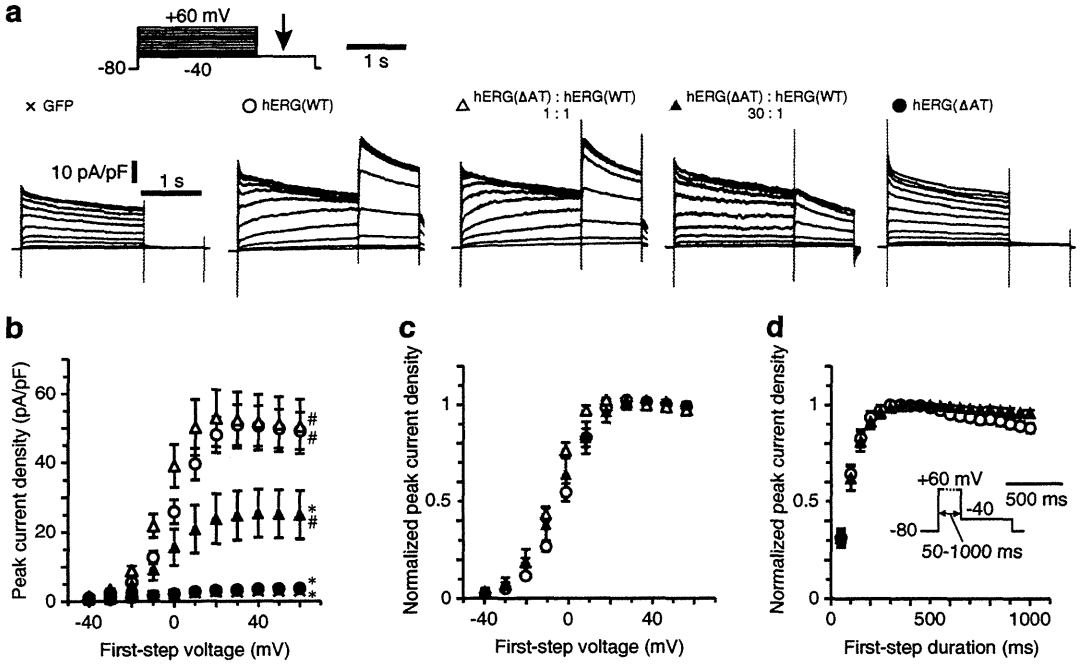

Fig. 6 Whole cell currents in HEK-293 T cells transfected with *hERG*(WT) and/or *hERG*(ΔAT). **a** Sample current responses of HEK-293 T cells transfected with the indicated genes. Currents mediated by hERG channels became evident as "tail" currents during the second step of the voltage stimulus protocol (*arrow* in schematic); n=18, 14, 14, 10, and 12 for cells transfected with *hERG*(WT), *hERG*(ΔAT), 1:1 and 30:1 mixtures of these genes, and the *GFP* gene alone (control), respectively. **b** Mean peak density of the tail current as a function of the first-step voltages. #P<0.001 (rank-sum test) compared with *GFP*-transfected cells; *P<0.001 (rank-sum test) compared with *hERG*(WT)-transfected cells. **c** Relative peak amplitude of the tail current as a function of the first-step voltage. **d** Activation time-course of hERG channels. Mean relative peak amplitude of the tail current elicited by a double-voltage step stimulus plotted as a function of the duration of the first-step voltage. In this experiment, the duration was varied as shown schematically in the *inset*

of the last exon–exon junction often undergo nonsense-mediated mRNA decay, which effectively reduces the amount of abnormal mRNAs [21]. We found that the level of transcripts from the *hERG*(ΔAT) allele was much lower than that for the *hERG*(WT) allele. One possibility suggested from this result is that *hERG*(ΔAT) mRNA might be degraded via the nonsense-mediated decay pathway.

The immunoblot analysis using HEK-293 T cells showed that hERG(ΔAT) produced lower protein levels than hERG(WT), regardless of whether the cells were co-transfected with hERG(WT) cDNA (Fig. 4). Confocal microscopy confirmed that the expression level of the hERG(ΔAT) subunit was much lower than that of the hERG(WT) subunit and showed that the slight amounts of mutant protein produced remained localized within the cells. One possibility arising from these results is that the reduced surface expression of the hERG(ΔAT) subunit simply reflects decreased protein production. Another possibility is that trafficking of hERG(ΔAT) is impaired, leading to decreased surface expression. Truncated proteins are often trafficking deficient, misfolded, misrouted, and consequently subjected to endoplasmic reticulum-associated degradation [22]. Therefore, both production and trafficking of the hERG(ΔAT) subunit may be impaired, but further study is required to gauge the relative influence of these two mechanisms.

Nonsense or frameshift mutations are located more frequently in the C-terminal region of hERG channel subunits than in the transmembrane pore domain (S5–loop–S6) [8]. The frameshift mutation we evaluated here is located in the S5-pore. Immunoblotting or immunohistochemistry experiments have confirmed that the Q725X [17] frameshift mutation in the C-terminus disrupts the tetrameric assembly of hERG and that E698X [23] and R863X [23, 24] lead to defective protein trafficking. Moreover, patch-clamp experiments have confirmed that these mutant proteins fail to produce functional homomeric channels and exert at most only a weak dominant-negative effect on WT proteins. Minigene-based experiments have demonstrated that hERG mutations such as P926Afs+14X [25], R1014X [26], R1005fs+50X, and Q1070X [27], all lead to degradation of the mutant mRNAs via the nonsense-mediated decay pathway. The functional consequence of the ΔAT frameshift mutation is therefore likely to be due to loss of functional hERG channels on the plasma membrane through multiple cellular mechanisms.

hERG(ΔAT)-transfected HEK-293 T cells were observed to lack a tail current, suggesting that hERG(ΔAT) subunits cannot form functional channels on their own. In contrast, cells co-transfected with *hERG*(ΔAT) and *hERG*(WT) did display a tail current. We surmise that this current is mediated largely by homomeric hERG(WT) channels, for the following reasons: First, the current amplitude decreased with the amount of *hERG*(WT) used in the transfection (Fig. 6b). Second, the cells transfected with a 30:1 mixture of *hERG*(ΔAT)/*hERG*(WT) displayed a tail current with voltage and time dependencies of activation similar to those of cells transfected with *hERG*(WT) only. The simplest interpretation of these findings is that the hERG(ΔAT) subunit contributes little to functional channel formation even in the presence of the hERG(WT) subunit. We cannot exclude the possibility that hERG(ΔAT) and hERG(WT) subunits associate to form functional heteromeric channels with small conductances. However, such heteromeric channels would most likely comprise only a small fraction of the total hERG channel population because hERG(WT) subunits appeared to greatly outnumber hERG(ΔAT) subunits on the cell surface (Fig. 5). Therefore, heteromeric channels would contribute little to whole cell electrical activity. This scenario may explain the mild cardiac symptoms of our patient, who was heterozygous for *hERG*(ΔAT). Furthermore, a homozygous *hERG*(ΔAT) carrier would most likely display severe symptoms resulting from a lack of functional hERG channels (Fig. 6a, b).

Our patient with the *hERG*(ΔAT) mutation displayed only mild symptoms during her everyday life and was categorized into an intermediate risk group in terms of her QTc interval, genotype, and gender. Nevertheless, she died from SCD 10 h after awakening from general anesthesia; her postoperative course was uneventful otherwise. The loss-of-function mutation in *hERG* identified in the current study might underlie the pathogenesis of her SCD. Patients with LQT2 are highly vulnerable to alterations in their physical and mental states that involve sympathetic surges [6]. For example, emotional stress, exercise, and various drugs [28] may trigger life-threatening arrhythmias in these patients. Our findings provide deeper insight into the genotype–phenotype relationship underlying LQT2 and a better understanding of the variability of the clinical presentation and subsequent risk stratification of LQTS in general.

Conclusion

We identified a novel *hERG* frameshift mutation (*hERG*(ΔAT)) in a patient who died from SCD. The ΔAT mutation decreased the number of functional hERG channels presumably by impairing the posttranscriptional and post-translational processing of the *hERG*(ΔAT) product. This decrease may explain, at least in part, the mild cardiac symptoms of our patient (who was heterozygous for *hERG*(ΔAT)) in everyday life and may have later contributed to her death.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethics statement The experiments described in this manuscript conform to the Declaration of Helsinki, and the protocols of gene sampling and manipulation were approved by the University of Toyama's committee on the usage of human genetic material (#24-1).

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RESEARCH Open Access

Increased interleukin-17 in the cerebrospinal fluid in sporadic Creutzfeldt-Jakob disease: a case-control study of rapidly progressive dementia

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Abstract

Background: Inflammatory responses in the cerebrospinal fluid (CSF) of patients with sporadic Creutzfeldt-Jakob disease (sCJD) remain elusive.

Methods: We conducted a case-control study, in which 14 patients with sCJD, 14 with noninflammatory neurological disorders, and 14 with autoimmune encephalitis were enrolled. We used the suspension array system to measure the concentrations of 27 cytokines in CSF. The cytokine titers of the three groups were compared, and the correlation between the relevant cytokine titers and clinical parameters was investigated in the patients with sCJD.

Results: Levels of the two cytokines interleukin (IL)-1 receptor antagonist and IL-17 were significantly elevated in the patients with sCJD compared with those in the patients with noninflammatory neurological disorders: IL-17 levels in sCJD were approximately ten times higher than in the noninflammatory neurological disorders (mean, 35.46 vs. 3.45 pg/ml; $P < 0.001$) but comparable to that in encephalitis (mean, 32.16 pg/ml). In contrast, levels of classical proinflammatory cytokines such as IL-12(p70) and tumor necrosis factor-α were increased only in encephalitis. Although not significant, IL-17 titers tended to be higher in the patients with shorter disease duration before CSF sampling ($r = -0.452$; $P = 0.104$) and in those with lower CSF total protein concentrations ($r = -0.473$; $P = 0.086$).

Conclusions: IL-17 is significantly increased in CSF in sCJD, which can be an early event in the pathogenesis of sCJD.

Keywords: Creutzfeldt-Jakob disease, Cytokines, Inflammation, Interleukin-17, Neurodegeneration

Background

Creutzfeldt-Jakob disease (CJD) is a rapidly progressive neurodegenerative disorder belonging to the human prion diseases and is characterized by the accumulation of prion or PrP^{Sc} [1]. Immunological processes have been poorly recognized in the central nervous system (CNS) of patients with CJD, partly because pleocytosis or increased protein levels are rarely observed in the cerebrospinal fluid (CSF) of patients with CJD. However, we have detected antibodies to N-methyl-D-aspartate receptors, typically present in autoimmune limbic encephalitis [2,3], in CSF of patients with CJD, which suggests inflammatory or autoimmune responses in the CNS of patients with CJD.

Cytokines are pivotal factors in CNS inflammation in autoimmune and neurodegenerative diseases [4]. The pathogenic activities of cytokines have been vigorously investigated in prion-infected animal models [5-7]. However, there is still uncertainty regarding cytokine abnormalities, particularly in patients with CJD, because some reports suggest an increase in levels of the proinflammatory cytokines [8,9] while others suggest an increase in levels of the anti-inflammatory cytokines [10,11] in CSF. Furthermore, levels of key cytokines in autoimmune and inflammatory neurological disorders, including interleukin (IL)-17 [12], have not been evaluated in patients with CJD. We systematically measured levels of multiple proinflammatory

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and anti-inflammatory cytokines in CSF of patients with CJD to detect CNS inflammatory responses that can be associated with the pathogenesis of CJD.

Methods

Clinical and neuropathological examination

A case-control study was conducted, and patients admitted to our hospitals from April 2004 to September 2012, who were diagnosed with sporadic CJD (sCJD) according to World Health Organization criteria, were recruited [13]. The prion protein gene (*PRNP*) was analyzed in the open reading frame after extracting DNA from the patients' blood samples [14,15]. Assays were performed to assess the CSF γ-isoform of the 14-3-3 protein (cut-off value, 500 μg/ml) [16] and total tau protein (cut-off value, 1,300 pg/ml) [17]. With regard to post-mortem examination, sections from formalin-fixed paraffin-embedded blocks of the brain were investigated. Mouse monoclonal antibody 3F4 (Dako, Glostrup, Denmark) and the EnVision amplified method (EnVision Plus kit; Dako) after hydrolytic autoclaving of the sections were used to perform immunohistochemical analysis for PrP [14,18]. Purification and Western blotting of protease-resistant PrP (PrP^{Sc}) from frozen cerebral cortical samples were performed as described in a previous study [19]. Typing of PrP^{Sc} (type 1 or type 2) was performed according to the reported classification system [20].

We enrolled age-matched (≥50 years) patients with noninflammatory neurological disorders as controls and age-matched patients with noninfectious or autoimmune encephalitis in whom polymerase chain reaction was negative for herpes simplex virus-1 and virus-2, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus and human herpes virus-6; autoantibodies were not necessarily screened. This study was approved by the ethics committee of the Tokushima University Hospital and was performed in accordance with the ethical standards described in the 1964 Declaration of Helsinki. Written informed consent was obtained from all participants (or guardians of participants) in the study. We obtained CSF samples by lumbar puncture at the time of evaluation from patients who were undergoing CSF examination as part of their diagnostic procedure; initial pressure, cell count, total protein level, and glucose level were measured. Remaining samples were frozen at −80°C until further investigation.

Cytokine assay

The Bio-Plex Pro Human Cytokine 27-plex assay (M50-0KCAF0Y; Bio-Rad, Hercules, CA) was used according to the manufacturer's instructions to evaluate the concentrations of 27 CSF cytokines and chemokines. The 27 cytokines of the panel were IL-1β; IL-1 receptor antagonist (IL-1ra); IL-2; IL-4; IL-5; IL-6; IL-7; IL-8/CXCL8; IL-9; IL-10; IL-12(p70); IL-13; IL-15; IL-17; eotaxin/CCL1; fibroblast growth factor-2 (FGF-2); granulocyte colony-stimulating factor (G-CSF); granulocyte macrophage colony-stimulating factor (GM-CSF); interferon-γ (IFN-γ); interferon-inducible protein-10 (IP-10)/CXCL10; monocyte chemotactic protein-1 (MCP-1)/CCL2; macrophage inflammatory protein (MIP)-1α/CCL3; MIP-1β/CCL4; platelet-derived growth factor BB (PDGF-BB); regulated on activation, normal T cell expressed and secreted (RANTES)/CCL5; tumor necrosis factor-α (TNF-α), and vascular endothelial growth factor (VEGF).

Statistical analysis

The Kruskal-Wallis test was used to compare the study groups, followed by Dunn's multiple comparison post-hoc analysis. In the multiple comparison analysis, P values <0.001 were considered to indicate statistical significance, and P values <0.05 were considered to indicate trends. Spearman's rank correlation coefficient was then used to assess the correlations between the cytokine titers and the duration before CSF sampling, the total protein concentrations, and the total tau protein titers. In the correlation analysis, P values <0.05 were considered to indicate statistical significance. GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) was used to analyze all the data.

Results

Clinical profiles

We enrolled 14 patients with sCJD (men, 5; age, 73.5 ± 4.9 years (mean ± standard deviation (SD))): seven with definite and seven with probable sCJD. *PRNP* was homozygous for methionine at codon 129 in all the patients. In addition, PrP^{Sc} was type 1 in five patients and type 2 in one patient. Moreover, the CSF cell count was normal in all the patients. Furthermore, the CSF 14-3-3 protein was positive in 12 of 14 patients, and the level of total tau protein was elevated in 10 of 13 patients (Table 1). The control subjects were 14 patients with noninflammatory neurological disorders (men, 10; age, 71.8 ± 10.9 years); eight had idiopathic normal pressure hydrocephalus, three had parkinsonism, two had myalgia/neuralgia and one had constant headaches. In addition, we enrolled 14 age-matched patients with noninfectious or autoimmune encephalitis (men, 8; age, 64.2 ± 7.5 years), including one with gastric cancer, one with colon cancer, one with prostate cancer, one with acoustic tumor, one with Hashimoto's thyroiditis, one with CNS lupus and schizophrenia, one with anti-voltage-gated potassium channel complex antibodies-associated limbic encephalitis, and seven without tumors or specific autoantibodies.

CSF cytokine profiles

Levels of IL-1ra and IL-17 were significantly higher in the patients with sCJD than in the controls; the IL-17

Table 1 Clinical profiles of patients with sporadic Creutzfeldt-Jakob disease

Number	Age, sex	Diagnosis	Codon 129, PrP^{Sc} type	Cell count (/μl), protein (mg/dl)	14-3-3 (μg/ml)	Tau (pg/ml)	Duration before sampling (months)
1	69, M	Probable	MM	3, 27	+	Not performed	1
2	74, F	Definite	MM	2, 27	+	1275	4
3	76, M	Probable	MM	6, 60	7313	>2400	2.5
4	70, F	Probable	MM	5, 43	+	8860	3
5	76, F	Probable	MM	1, 49	23503	17340	3
6	74, F	Probable	MM	4, 47	8688	22750	2
7	75, M	Probable	MM	2, 25	0	0	1
8	83, F	Definite	MM1	1, 27	2244	10290	6
9	65, F	Probable	MM	0, 25	1256	3620	8
10	80, M	Definite	MM1	1, 87	15226	8520	22
11	74, M	Definite	MM1	1, 30	697	1707	28
12	74, F	Definite	MM1	2, 27	531	1930	10
13	73, F	Definite	MM1	1, 35	7188	>13000	4
14	66, F	Definite	MM2-C	1, 64	0	1180	13

C, cortical type; MM, homozygous for methionine.

titers in the patients with sCJD were comparable to those in the patients with encephalitis and approximately ten times higher than those in the control subjects. The remaining cytokine levels were not significantly elevated in sCJD, including the classical proinflammatory cytokines IL-1β and TNF-α and the anti-inflammatory cytokines IL-4 and IL-10. The elevation of IL-12(p70), FGF-2, PDGF-BB, and TNF-α levels in the patients with encephalitis indicated clear contrast with the normal findings in those with sCJD (Table 2 and Figure 1).

Correlation between interleukin-17 titers and clinical profiles of Creutzfeldt-Jakob disease

We assessed the relationships between IL-17 titers and the clinical data of sCJD to investigate how elevated IL-17 levels were associated with disease progression. The IL-17 titers tended to be higher in the patients with shorter disease duration before CSF sampling (Spearman's $r = -0.452$; $P = 0.104$), which suggested that levels of IL-17 are increased in the early phases of the disease when neuronal damage is less severe (Figure 2A). Although not significant, the IL-17 titers tended to be higher when the CSF total protein concentrations were lower (Spearman's $r = -0.473$; $P = 0.086$; Figure 2B). There was no significant correlation between IL-17 titers and total tau protein titers (Spearman's $r = -0.236$; $P = 0.485$), elevation of which may reflect the rate of neurodegeneration.

Discussion

In CSF of the patients with sCJD, we detected increases in IL-17, a cytokine that induces inflammation and can be pathogenic for autoimmune neurological disorders, such as multiple sclerosis and experimental autoimmune encephalomyelitis (EAE) [12,21,22]. It is important to note that IL-17 is associated with enhanced neuroinflammation in the absence of PrP^C, because PrP^C downregulation can contribute to the pathogenesis of prion disease. PrP^C-knockout exacerbates and prolongs neuroinflammation in EAE and is accompanied by an increase in IFN-γ and IL-17 mRNA in CNS [23]. Moreover, PrP^C-knockout can lead to enhanced EAE severity but with reduction of IL-17-positive Th17 cells [24]. These findings suggest that PrP^C downregulation enhances neuroinflammation and IL-17 production, but that IL-17-producing cells differ from Th17 cells. Besides Th17, IL-17 can be produced by glial cells in CNS [21], and astrocytes are critical for the IL-17 pathways in EAE [25,26]. Furthermore, IL-17 can be expressed by microglia in response to IL-1β or IL-23 [27]. Considering the rarity of T cells in CNS of patients with CJD, astrocytes and microglia can be major sources of IL-17 in CJD.

Increased IL-17 production is a new finding in prion disease, whereas the activities of other cytokines have been investigated in prion-infected mice and to a lesser extent in patients with CJD. Cytokines that are overexpressed in prion-infected mouse brain include TNF-α, IL-1α, IL-1β, IL-6, IL-12(p40), transforming growth factor-β1, MCP-1/CCL2, RANTES/CCL5, CXCL1, IP-10/CXCL10 and CXCL13 [5-7]. However, CSF findings in patients with CJD remain controversial; early studies reported an increase in levels of proinflammatory cytokines TNF-α and IL-1β [8,9], but recent studies

Table 2 Cytokine profiles in the cerebrospinal fluid

Cytokine (pg/ml)	Control		CJD		Encephalitis		P values (vs. Control)	
	Mean	SD	Mean	SD	Mean	SD	CJD	Encephalitis
IL-1β	0.2371	0.2433	0.4871	0.4529	0.1471	0.2015	NS	NS
IL-1ra	0	0	18.43	12.38	18.49	13.81	<0.001	<0.001
IL-2	0.6736	1.068	0.7757	1.007	0.1200	0.3007	NS	NS
IL-4	0.1800	0.0854	0.5843	0.6772	0.3207	0.3526	NS	NS
IL-5	0.4686	0.3360	0.2250	0.3128	0.5514	0.5287	NS	NS
IL-6	5.911	1.834	12.36	28.79	14.48	16.42	NS	NS
IL-7	0.3593	0.5244	1.196	2.301	0.9507	0.9143	NS	NS
IL-8/CXCL8	57.38	14.30	78.63	49.37	130.9	115.9	NS	<0.05
IL-9	10.80	2.974	11.06	6.014	34.32	55.89	NS	<0.01
IL-10	1.178	0.5076	1.246	1.300	2.871	3.246	NS	NS
IL-12(p70)	0.4243	0.5698	1.656	1.790	5.481	8.797	NS	<0.001
IL-13	3.204	2.282	2.081	1.845	5.210	7.876	NS	NS
IL-15	5.691	4.372	6.366	4.853	20.19	13.01	NS	<0.05
IL-17	3.454	7.814	35.46	44.40	32.16	27.14	<0.001	<0.001
Eotaxin/CCL11	0	0	3.236	5.317	3.117	7.441	NS	<0.05
FGF-2	7.244	4.743	15.72	12.14	55.16	31.04	NS	<0.001
G-CSF	6.539	2.477	10.92	7.283	13.51	8.212	NS	<0.05
GM-CSF	339.6	30.78	166.2	103.9	184.7	139.6	<0.01	<0.01
IFN-γ	9.297	3.523	21.33	32.72	17.13	14.67	NS	NS
IP-10/CXCL10	1299	342.5	1602	866.9	6140	8906	NS	<0.05
MCP-1/CCL2	318.2	118.9	372.7	425.8	357.8	164.2	NS	NS
MIP-1α/CCL3	0.7614	0.3028	0.4243	0.8461	2.481	3.056	<0.05	NS
MIP-1β/CCL4	27.38	10.65	31.19	17.70	44.48	24.73	NS	NS
PDGF-BB	1.196	3.278	3.638	3.016	16.24	24.69	NS	<0.001
RANTES/CCL5	3.168	3.691	4.958	4.583	6.134	5.396	NS	NS
TNF-α	6.481	1.837	13.17	10.79	40.68	23.12	NS	<0.001
VEGF	12.88	2.234	9.086	6.164	18.51	17.16	NS	NS

CJD, Creutzfeldt-Jakob disease; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL, interleukin; IL-1ra, IL-1 receptor antagonist; IP-10, interferon-inducible protein-10; MCP-1, monocyte chemotactic protein-1; MIP, macrophage inflammatory protein; NS, not significant; PDGF-BB, platelet-derived growth factor BB; RANTES, regulated on activation, normal T cell expressed and secreted; SD, standard deviation; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

demonstrated normal levels of these classical proinflammatory cytokines and increased levels of the anti-inflammatory cytokines IL-4 and IL-10 [10,11]. In addition, these studies reported elevated levels of IL-8/CXCL8 [11], a chemokine that can be associated with the production of IL-17 in opticospinal multiple sclerosis [28]. Of these, IL-1 can be pathogenic for prion disease because IL-1 receptor knockout mice demonstrate significantly longer prion-incubation times than controls [6]. In contrast, IL-10 can be neuroprotective because IL-10 knockout mice demonstrate significantly shorter incubation times [6,29]. Conversely, TNF-α, IL-4, IL-6, IL-12(p40), IL-12(p35), IL-13, and transforming growth factor-β1 are probably not pathogenic in prion disease because knockout of these cytokines did not change the disease course of prion-inoculated mice [5,6,29-31].

Our results were partly consistent with those of recent reports on human CSF cytokines quantified using enzyme-linked immunosorbent assay kits that revealed low titers of IL-1β, IL-6, IL-12, and TNF-α in patients with CJD [10,11]. However, the significant elevations of IL-4, IL-10, and IL-8/CXCL8 levels in CJD reported in these studies [10,11] were not observed in this study. This inconsistency may result from the different methodologies and disease controls and should be further investigated in future.

Our results imply that elevated levels of IL-17, a pro-inflammatory cytokine, can be an early event in sCJD

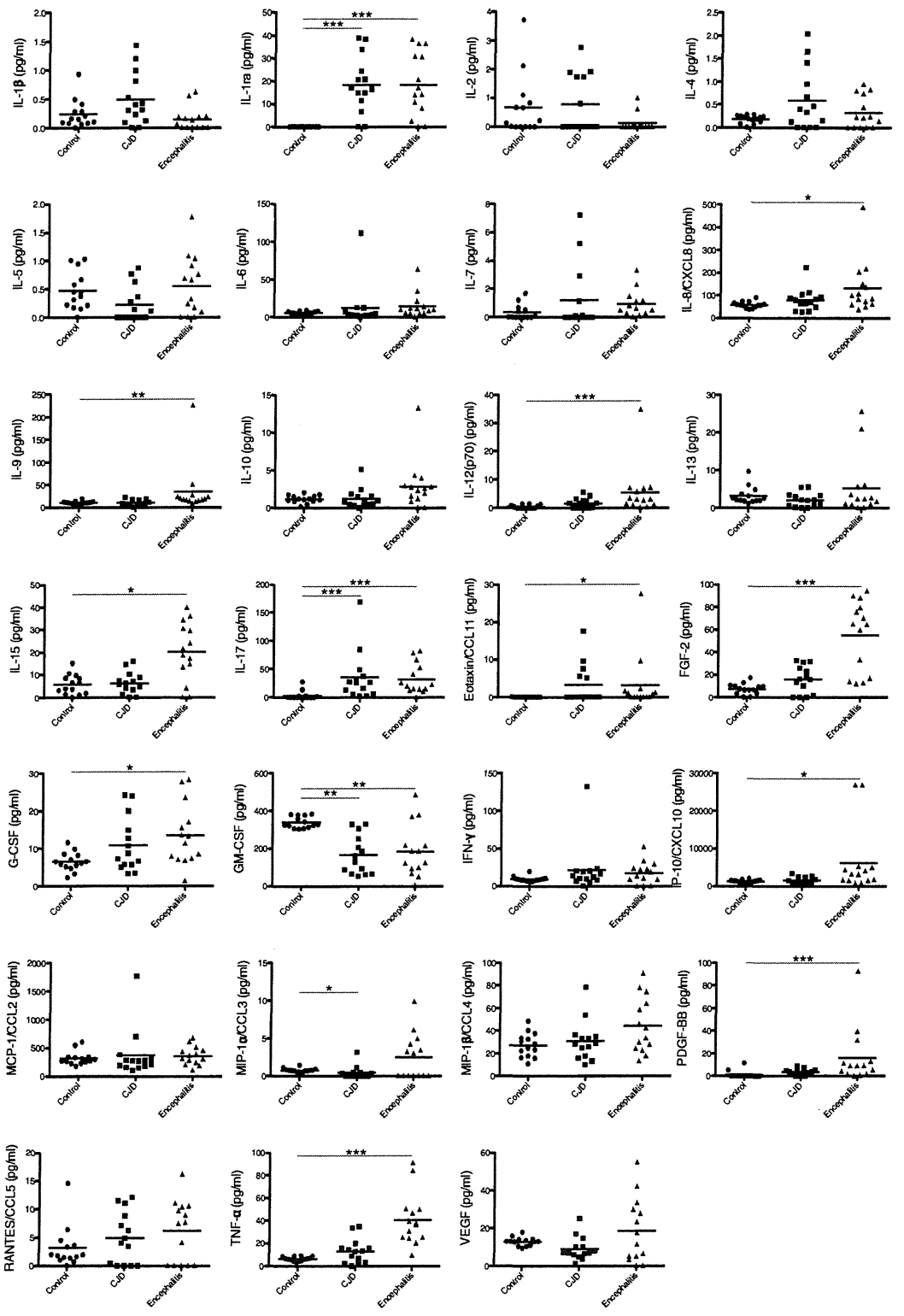

Figure 1 Cytokines titers of the multiplex assay. Cytokine titers of the control subjects ($n = 14$), the patients with sporadic Creutzfeldt-Jakob disease (CJD, $n = 14$) and those with autoimmune encephalitis ($n = 14$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

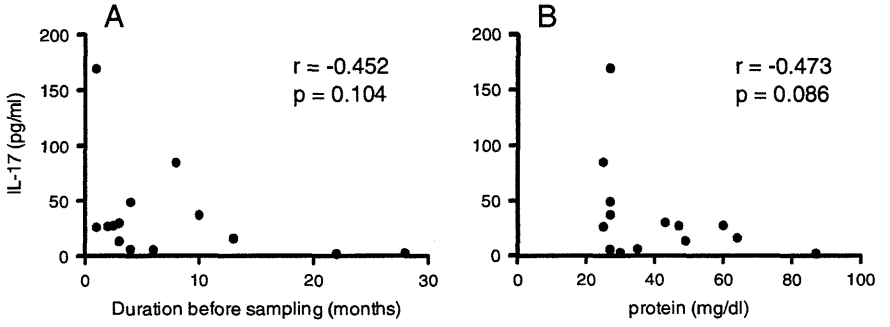

Figure 2 Correlation of cytokine titers and clinical parameters. (A) Correlation between cerebrospinal fluid interleukin-17 (IL-17) titers and disease duration before sampling in the patients with sporadic Creutzfeldt-Jakob disease. **(B)** Correlation between cerebrospinal fluid IL-17 and total protein titers in the patients with sporadic Creutzfeldt-Jakob disease. Total protein titer was used as a general inflammatory marker in the central nervous system.

rather than a mere consequence of neurodegeneration later in the disease course. This observation is intriguing because the functions of inflammation in the early stages of pathology have been vigorously investigated in several neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis [32]. Moreover, it is worth noting that inflammatory responses may have dual functions in neurodegeneration, both as instigators of damage and as guardians of brain homeostasis. Therefore, investigation of the potential activities of IL-17 and other inflammatory markers [33,34] is warranted in prion disease.

Abbreviations

CJD: Creutzfeldt-Jakob disease; CNS: central nervous system; CSF: cerebrospinal fluid; EAE: experimental autoimmune encephalomyelitis; FGF-2: fibroblast growth factor-2; G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte macrophage colony-stimulating factor; IFN-γ: interferon-γ; IL: interleukin; IL-1ra: IL-1 receptor antagonist; IP-10: interferon-inducible protein-10; MCP-1: monocyte chemotactic protein-1; MIP: macrophage inflammatory protein; PDGF-BB: platelet-derived growth factor BB; *PRNP*: prion protein gene; RANTES: regulated on activation, normal T cell expressed and secreted; sCJD: sporadic Creutzfeldt-Jakob disease; SD: standard deviation; TNF-α: tumor necrosis factor-α; VEGF: vascular endothelial growth factor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KF participated in the design of the study, acquisition and analysis of data, statistical analysis and manuscript drafting and revision. NM, TY, and RK participated in data analysis and manuscript revision. YT, Y Iwasaki, and MY participated in data acquisition and manuscript revision. Y Izumi participated in acquisition and analysis of data and manuscript revision. All the authors read and approved the final manuscript.

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Review article

Anti-NMDAR autoimmune encephalitis

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Abstract

The *N*-methyl-D-aspartate receptor (NMDAR) is involved in normal physiological and pathological states in the brain. Anti-NMDAR encephalitis is characterized by memory deficits, seizures, confusion, and psychological disturbances in males and females of all ages. This type of encephalitis is often associated with ovarian teratoma in young women, but children are less likely to have tumors. Anti-NMDAR encephalitis is a neuroimmune syndrome in patients with autoantibodies recognizing extracellular epitopes of NMDAR, and the autoantibodies attenuate NMDAR function through the internalization of NMDAR. Following the initial symptoms of inflammation, the patients show the various symptoms such as memory loss, confusion, emotional disturbances, psychosis, dyskinesis, decrease in speech intelligibility, and seizures. About half of these patients improved with immunotherapy including high-dose intravenous corticosteroids and intravenous immunoglobulins is administrated to these patients, but the patients who had no improvement with these therapy require further treatments with rituximab or cyclophosphamide. It is necessary to detect anti-NMDAR antibodies at early stages, because the prognosis of these patients may be improved by early treatment. Recovery is slow, and the patients may have some disturbances in their motor function and cognition. The pathologic mechanism underlying the development of anti-NMDAR encephalitis has been elucidated gradually, but the optimal treatment has not yet been clarified. Further studies are required to clarify in detail the mechanism underlying anti-NMDA encephalitis and to develop effective treatments.

Keywords: Encephalitis; *N*-Methyl-D-aspartate receptor; Autoantibody

1. Introduction

The *N*-methyl-D-aspartate receptor (NMDAR) is critically involved in normal neural network formation, synaptic plasticity, and higher brain functions such as learning and memory [1,2]. A highly active NMDAR is composed of multiple glutamate-binding GluRε (NR2, GluN2) subunits and a glycine/D-serine-binding GluRζ1 (NR1, GluN1) subunit [3]. The hyperactivation of NMDAR has been shown to mediate acute neuronal death and chronic neurodegeneration [4]. In contrast, the hypoactivation of NMDAR is involved in the development of psychiatric states [5,6]. The NMDAR subunits are widely distributed throughout the brain including the limbic system. In situ hybridization analyses, the GluRζ1 (NR1, GluN1) subunit mRNA distributes ubiquitously in the brain. The GluRε1 (NR2A, GluN2A) subunit mRNA is expressed postnatally and widely in the brain. The GluRε2 (NR2B, GluN2B) subunit mRNA is found throughout the entire embryonic brain, but its expression becomes restricted to the forebrain at postnatal stages. The GluRε3 (NR2C,

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GluN2C) subunit mRNA appears postnatally and predominantly in the cerebellum. The GluRε4 (NR2D, GluN2D) subunit mRNA is abundantly expressed in the diencephalon and the brainstem at embryonic and neonatal stages [3].

Limbic encephalitis is an inflammation of the limbic system, which includes the hippocampus, thalamus, hypothalamus, and amygdala. The symptoms of limbic encephalitis are memory deficits, seizures, confusion, and psychological disturbances. In 1960, the disease was first described as subacute encephalitis affecting the limbic areas by Brierley et al. [7]. Subsequently, it was mainly reported as paraneoplastic limbic encephalitis associated with lung carcinoma and malignancies in the ovary, breast, stomach, uterus, kidney, bladder, and colon [8]. The report suggested that limbic encephalitis is caused by autoimmunity against limbic system antigens, similarly to Eaton–Lambert syndrome. In 2001, Buckley et al. reported the cases of two patients with limbic encephalitis in whom the antibodies to voltage-gated potassium channels (VGKCs) were detected in their serum samples [9]. Later, the true target antigen of the antibodies to VGKCs has been shown to be leucine rich glioma inactivated 1 (LGI1) and contactin associated protein 2 (CASPR2) [10]. Furthermore, anti-NMDAR NR2 subunit autoantibodies were detected in some patients with acute encephalitis including those with limbic encephalitis in 2003 [11]. From these reports, the role of immunity and inflammatory processes in epilepsy or encephalitis became the focus of interest.

In 2007, the concept that anti-NMDAR encephalitis associated with ovarian teratoma, a severe, potentially lethal, treatment-responsive disorder, is mediated by autoantibodies against NMDAR was proposed by Dalmau et al. [12]. However, an increasing number of cases have been reported for both men and women from children to adults of advanced age, with and without tumors [13–18]. Recently, the spectrum of the neuroautoimmune syndromes has greatly expanded by the discovery of new antigen-specific antibodies. These syndromes are suggested to categorize (1) classical paraneoplastic syndromes associated with antibodies to intracellular antigens such as Hu, Ma2, collapsin-responsive mediator protein-5 (CRMP5), Yo or amphiphysin and (2) autoimmune encephalitis associated with antibodies to cell surface or synaptic antigens such as NMDAR, gamma aminobutyric acid receptor (GABAR-B), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), LIG1 or CASPR2, based on the underlying immunopathogenesis and responsiveness to immunotherapy [19,20]. In this report, we present the case of a girl with anti-NMDAR encephalitis and review the clinical presentations, diagnosis, and evidence supporting autoimmune mechanisms of this syndrome.

2. Case presentation

A 7-year-old previously healthy Japanese girl had a cough and low-grade fever. 5 days later, she sought her mother frequently and complained of anxiety. A week later, she was brought to our hospital owing to

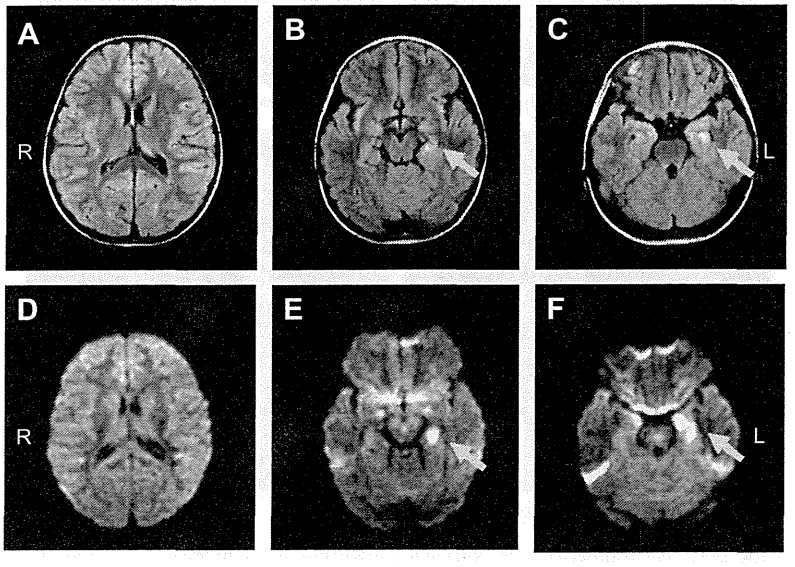

Fig. 1. MR images of a patient with anti-NMDAR encephalitis. Axial FLAIR images (panels A–C) and diffusion-weighted (DW) images (panels D–F) of a 7-years-old girl with anti-NMDAR encephalitis. Arrows in panels B, C, E, and F show hyperintensities in the left medial temporal lobe on FLAIR and DW images.

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vomiting and her state of confusion. She had a temperature of 38.0 °C without focal neurologic deficits or meningeal signs. Her white blood cell count and serum C reactive protein level were slightly elevated. Cerebrospinal fluid (CSF) analysis revealed a lymphocytic pleocytosis of 296 nucleated cells/mm^3 with 90% lymphocytes and normal glucose and protein levels. Treatments with acyclovir and dexamethasone were started for presumed viral encephalitis. Her blood and CSF bacterial cultures showed negative results. Viral cultures and polymerase chain reaction analysis of CSF for herpes simplex virus also showed negative results. Thus, these treatments were stopped. Brain computerized tomography (CT) and magnetic resonance imaging (MRI) findings were normal. Electroencephalography (EEG) revealed diffuse slowing of waves, but no epileptic discharges.

Two days after admission, her state of confusion worsened and she spoke incomprehensible words, "blue, blue, blue" or "green, green, green" to her parents. She was treated with intravenous methylprednisolone, which did not improve her neurological states. She refused to take anything by mouth, necessitating nutritional support using a nasogastric tube. She demonstrated orofacial dyskinesias and involuntary movements of the right upper extremity on arousal. A week later, a second brain MRI with fluid attenuation inversion recovery (FLAIR) and diffusion-weighted imaging (DWI) revealed hyperintensities in the left medial temporal lobe (Fig. 1). A course of intravenous immunoglobulins (2 g/kg) was completed with no response. She began to develop symptoms of dyskinesias, stereotyped motor automatisms, and spastic rigidity. We had to administer sedatives and antipsychotic medications, because she was awake during nighttime and slept during daytime and her serum creatinine kinase level increased owing to her involuntary movements.

Two months later, she showed gradual improvements in her motor and cognitive functions. 3 months after her admission, she could take food by mouth and walk a short distance by herself. After her discharge, her serum was found to be positive for anti-NMDAR antibodies (Fig. 2) and we diagnosed her as having anti-NMDAR encephalitis. Presently, she goes to school cheerfully, but has some cognitive problems, such as mild memory disturbance and learning disabilities.

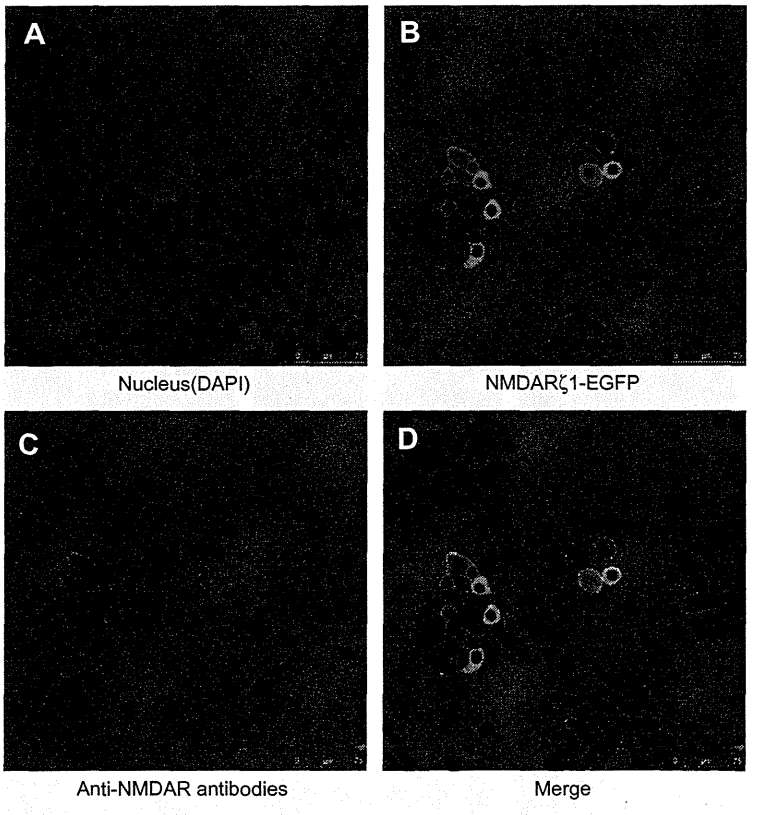

Fig. 2. Immunostaining of HEK 293T cells transfected with NMDAR ζ1-EGFP/ε2 subunits with serum from the 7-years-old girl with the anti-NMDAR encephalitis. Signals of nuclei DAPI (blue, A), fluorescence signals of NMDAR ζ1-EGFP (green, B), and immunofluorescence signals of autoantibodies in serum (magenta, C) from the patient. Panel D shows the merged image of these signals. Scale bar: 75 μm.

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3. Clinical features

The exact incidence of anti-NMDAR encephalitis is unclear. The antibodies to NMDAR were detected in 4% of patients with encephalitis by a multicenter, population-based prospective study in England [21]. In another study, anti-NMDAR antibodies were detected in 1% of patients with encephalitis of unknown etiology admitted to the intensive care unit [15]. In a study in the United Kingdom and Europe, 11.1% of patients suspected of having encephalitis were positive for anti-NMDAR antibodies [22]. In the California Encephalitis Project, 4% of patients with encephalitis of uncertain etiology aged <30 years were positive for anti-NMDAR antibodies [23]. From these studies, anti-NMDAR encephalitis is not rare, and may be misdiagnosed as an unidentified encephalitis or a psychiatric disorder [16,24–26]. Initially described as paraneoplastic encephalitis associated with ovarian teratoma, young female patients often had ovarian teratoma [12,17,22]. The other tumors were sex-cord stromal tumor, neuroendocrine tumor, teratoma of the mediastinum, small cell lung cancer, and lymphoma [17,18,22]. Recent reports of the expression of the NMDAR in some tumors and its role in tumor invasion are interesting [27–29]. Many cases in children are less likely to have tumors [13,14,18]. Anti-NMDAR encephalitis commonly occurs in young females, but has been reported in males and females of all ages (from 8 months to 85 years) [13,16,18,22].

Prodromal symptoms such as fever, headache, upper respiratory symptoms, vomiting, and diarrhea are observed in 48–86% of patients within 2 weeks before hospital admission [14,17,30]. The initial symptoms of anti-NMDAR encephalitis are evenly distributed between psychotic and neurologic. However, the severity and sequence of the symptoms such as memory loss, confusion, emotional disturbances, psychosis (delusions and hallucinations), dyskinesis, decrease in speech intelligibility, and seizures vary [14,17,18]. During the course of the disorder, 76–77% of patients have seizures, most commonly tonic–clonic seizures [14,17]. Patients presenting with psychosis are often treated with antipsychotic agents [16,24–26]. Sequentially, dyskinesis (especially orofacial), involuntary movement, spastic rigidity, echolalia, ataxia, refractory seizures, and decreased level of consciousness are observed [14,17,30,31]. Some patients develop drastic involuntary movements and spastic rigidity, and have high levels of creatine kinase [17,31]. Days–weeks later, autonomic instability often causes cardiac arrhythmia, hypotension, and central hypoventilation, requiring intubation or pacemakers [14,17]. Patients show gradual improvement in motor and cognitive functions. The median duration of hospitalization is in the range of 2–2.5 months (range, 1–14 months) [17,30].

Results of conventional investigations including examination of CSF, brain imaging, and EEG are nonspecific for anti-NMDAR encephalitis. CSF analysis revealed lymphocytic pleocytosis in many cases (68–91%), oligoclonal banding and increased CSF protein level within the first few days after the onset of neurological symptoms [17,22,30]. EEG demonstrated epileptic discharge or slowing of waves [17,22,30]. A unique electrographic pattern "Extreme delta brush" may be associated with a more prolonged illness [30,32]. There are some reports that EEG showed generalized rhythmic delta activity with a nonconvulsive status epilepticus [33,34]. In MRI, few patients showed hyperintensities on T2-weighted sequences or FLAIR images of the medial temporal lobes, corpus callosum, or cerebral cortex [17,18,22,30]. The results of brain PET were limited, but all the patients showed abnormal frontotemporal, occipital, and cerebellar hypermetabolism [22,35]. The identification of anti-NMDAR antibodies is critical for the diagnosis of anti-NMDAR encephalitis, because other clinical examination results are nonspecific.

4. Detection of anti-NMDAR antibodies

The laboratory approach to the detection of anti-NMDAR antibodies involves indirect or direct examinations. Indirect immunofluorescence on cryopreserved sections or primary cell cultures of the rodent brain may be a good screening test in patients suspected of having autoimmune encephalitis regardless of having autoantibodies for brain antigens or not [12,22]. The lysates of human embryonic kidney (HEK) cells ectopically expressing NR1 or NR1–NR2 heteromers and the peptide of the NMDAR subunit were used in in vitro enzyme-linked immunosorbent assay (ELISA) [17]. A cell-based assay is an immunoassay of culture cells (i.e., HEK cells) transfected with the complementary DNA (cDNA) representing the single or assembled NR1–NR2 subunits [12,36]. The cell-based assay is a more specific and sensitive evaluation system for detecting autoantibodies recognizing conformational extracellular epitopes of NMDAR [12,36].

Rapid quantitative evaluation systems for detecting autoantibodies against extracellular epitopes of NMDAR are necessary, because paraneoplastic anti-NMDAR encephalitis has a better prognosis after tumor resection and immunotherapy (corticosteroids, intravenous immunoglobulins, or plasma exchange) [12,17,18]. Thus, the establishment of cells stably expressing functional NMDAR is desirable. However, Ca^{2+} influx through NMDAR activated by glutamate and glycine present in a culture medium is toxic to non-neurons [37]. We reported a method to rapidly analyze the presence and function of autoantibodies against NMDAR using cultured cells (HEK293T) that stably expressed mutant NMDAR with decreased Ca^{2+}

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permeability on a heterologous cell surface [36]. The level of the anti-NMDAR antibody in serum of the patients is significantly higher than that in the CSF [13,22,38].

5. Treatment and prognosis

A randomized controlled trial of the treatment for anti-NMDAR encephalitis has not been reported. When the patients were diagnosed as having anti-NMDAR encephalitis, the immunotherapy including high-dose intravenous corticosteroids, intravenous immunoglobulins, plasma exchange, cyclophosphamide, azathioprine, mycophenolate mofetil, tacrolimus, methotrexate, and monoclonal antibodies (e.g., rituximab) was used in sequence or in combination [14,17,18]. Although a few patients recovered to their normal state with supportive care alone, most of the patients required further treatments such as tumor resection and immunotherapy [17,18,39]. Thus, Dalmau et al. proposed that the tumor (an ovarian teratoma or a testicular tumor) should be removed when present [39]. When tumor is not present, they prefer the first-line therapy with intravenous immunoglobulins, methylprednisolone, plasma exchange [40],

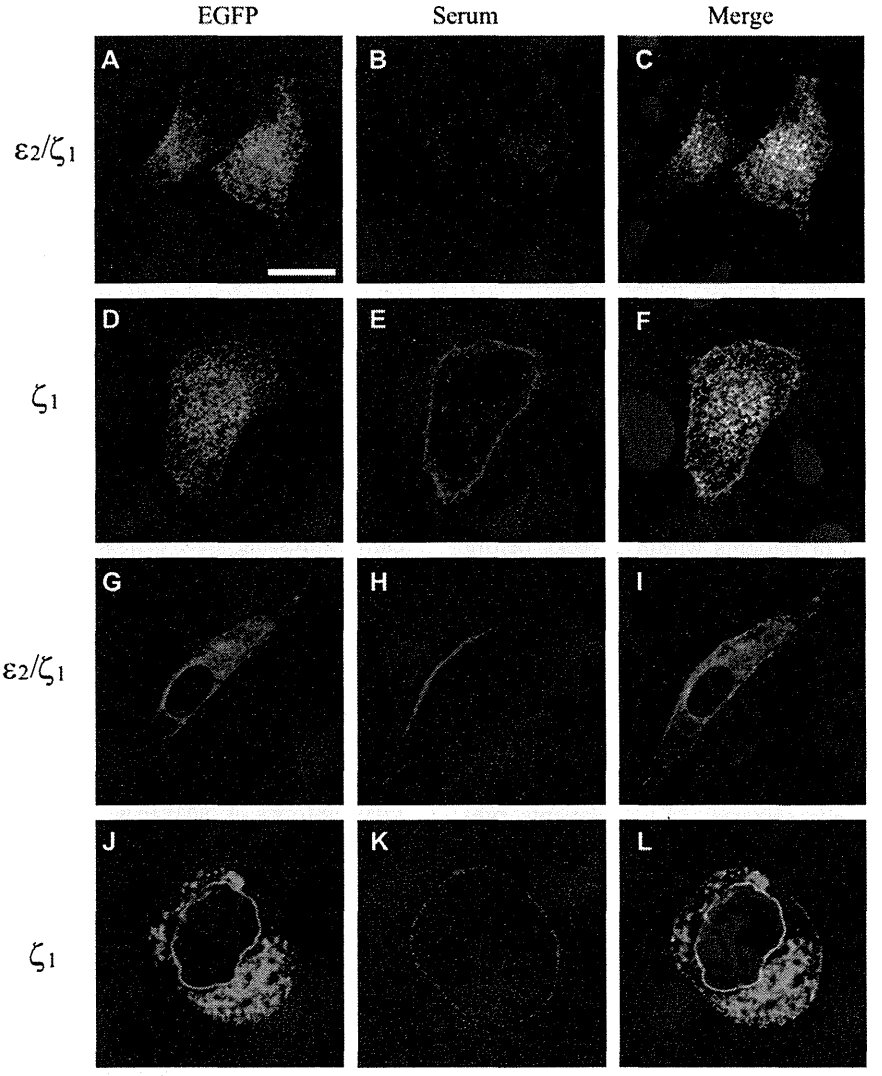

Fig. 3. Internalization of cell surface NMDAR subunits in Chinese hamster ovary (CHO) cells induced by patient's antibodies (reproduced from [36]). The ε2/ζ1-(A–C and G–I) and ζ1- (D–F and J–L) subunit-transfected CHO cells were incubated with the patient's antibodies at 37 °C (A–F) or 4 °C (G–L) then fixed and observed by confocal laser microscopy. The fluorescence signal of EGFP (green; A, D, G, and J) and the immunofluorescence signals of the antibodies in the serum (magenta; B, E, H, and K) samples from patients were detected and merged with the signal of DAPI (blue; C, F, I, and L). Scale bar: 25 μm.

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or their combinations. If no response is observed after 10 days, they prefer to start the second-line therapy with rituximab [41] or cyclophosphamide [42]. The patients who did not improve with the first line immunotherapy may have better outcome due to the second line immunotherapy [18]. For patients showing a good response, the treatment shifts to supportive care and tumor surveillance. The level of anti-NMDAR antibodies in CSF and serum usually decreases when patients show substantial clinical recovery [15,17,22,43].

Recovery may take 2 years or longer, and the patient may not always return to their former levels of motor function and cognition [14,17,18,44,45]. In a cohort of 252 patients, 81% experienced complete or near-complete recovery (Modified Rankin scale scores of 1–2) and 10% (14/252) died [18]. Relapses have been reported to occur in 20–30% of patients [14,17,18,22,46], and the occurrence is higher in patients without immunotherapy [18,46]. This finding suggests the benefit of early immune suppression and tumor resection.

6. Pathology

The clinical features of anti-NMDAR encephalitis correspond to the state caused by the change in the activity of NMDAR. Anti-NMDAR encephalitis is considered to be antibody-mediated because anti-NMDAR antibodies are detected in the serum or CSF of most patients, anti-NMDAR encephalitis has a better prognosis after tumor resection and immunotherapy, and antibody levels are related to clinical outcomes [15,17,22,43]. Furthermore, the reversibility of the disorder, irrespective of the duration of symptoms, suggests an immune-mediated neuronal dysfunction rather than irreversible degeneration [17,47].

These features indicate that anti-NMDAR antibodies do not mediate neuronal death by hyperactivation of NMDAR or complement or cytotoxic T-cell mechanisms, but that these antibodies recognize extracellular epitopes of NMDAR and change NMDAR functions. The mechanisms underlying the pathogenic effects were proposed, including attenuation of NMDAR function by internalization and degradation of NMDAR by anti-NMDAR antibodies associated with paraneoplasms, such as ovarian tumors [36,39,47]. The internalization of NMDAR with anti-NMDAR antibodies was suggested by a biochemical study and a study using primary cultured neurons [36,47]. We detected the immunofluorescence signals of autoantibodies in the cytoplasm in addition to the membrane in cells expressing NMDAR (Fig. 3) [36]. The antibodies in patients with anti-NMDAR encephalitis lead to the loss of surface NMDAR by antibody-mediated internalization, resulting in the attenuation of NMDAR function [39,47]. In anti-NMDAR encephalitis, autoantibodies crossreacting with NMDAR are produced against tumors or pathogens (*Mycoplasma pneumonia* [14], influenza viruses A and B, *Chlamydophilia pneumoniae*, *Bordetella pertussis*, *Bordetella parapertussis* [15], and Epstein–Barr virus [48]). Anti-NMDAR antibodies may be produced in subarachnoid space, because the patients with NMDAR encephalitis had significant B-cell expansion in CSF and infiltration of plasma cells around vessels or in CSF [17,49–51]. This intrathecal production of autoantibody may be important factor associated with poor prognosis or resistant to first-line immunotherapy with intravenous immunoglobulins, methylprednisolone, plasma exchange. On the other hand, it is also considered that the leakage of antibodies from vessels into the brain occurs in the patients,

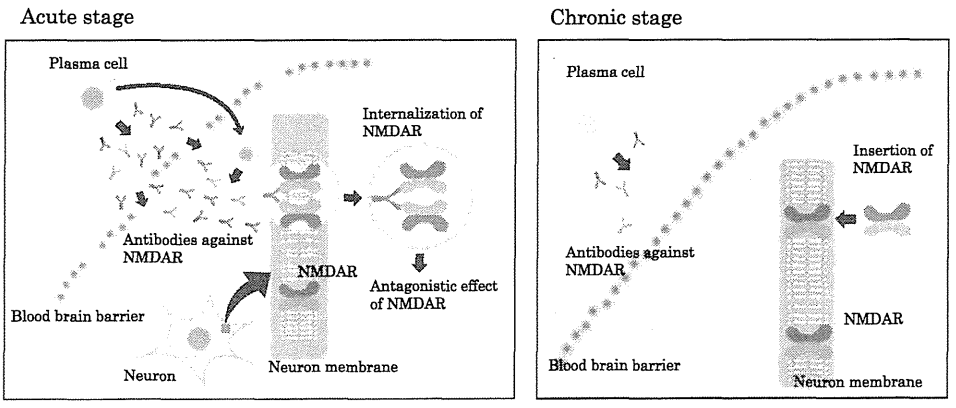

Fig. 4. Schematic models of the possible mechanism underlying the development of anti-NMDAR autoimmune encephalitis. At the acute stage, autoantibodies against NMDAR leak into CSF through the disruption of the blood–brain barrier. Intrathecal production of anti-NMDAR antibodies are also suggested. Anti-NMDAR antibodies induce the internalization of NMDAR. The decrease in the expression level of neuronal surface NMDAR results in neuronal hypoactivity. At the chronic stage, after the level of anti-NMDAR antibodies produced by plasma cells decreases and the blood–brain barrier is restored, the level of anti-NMDAR antibodies in CSF decreases. The NMDAR is expressed on the neuronal surface again, and neuronal functions recovers.

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because there are some reports that the anti-NMDAR antibody levels in serum are significantly higher than that in CSF [13,22,38]. Finally, anti-NMDAR antibodies in CSF disrupt the interactions between EPHB2R and NMDARs [52], which results in the attenuation of NMDAR function through the internalization of NMDAR (Fig. 4). At the chronic stage, after the level of anti-NMDAR antibodies produced by plasma cells decreases and the blood–brain barrier is restored, the level of anti-NMDAR antibodies in CSF decreases. The NMDAR is expressed on the neuronal surface again, and neuronal functions recovers (Fig. 4).

7. Conclusion

Anti-NMDAR encephalitis with central nerve or psychiatric symptoms is detected in a significant percentage of patients with acute encephalitis. The early and precise examination for the presence of anti-NMDAR antibodies is necessary, because the prognosis of these patients may be improved by early treatment. The pathologic mechanism underlying the development of anti-NMDAR encephalitis has been elucidated gradually in in vitro studies, but the optimal treatment has not yet been clarified. Further studies are required including those of additional cases and animal models of anti-NMDAR encephalitis to clarify in detail the mechanisms underlying the development of anti-NMDA encephalitis.

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