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Repeated follow-up of AQP4-IgG titer by cell-based assay in neuromyelitis optica spectrum disorders (NMOSD)

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ABSTRACT

Introduction: Neuromyelitis optica spectrum disorder (NMOSD) is characterized by the presence of serum anti-aquaporin 4 (AQP4) antibody. However, the significance of changes in the serum titer as a marker of disease severity or relapse prediction is unknown.

Methods: We collected clinical data and serum antibody titers by cell-based assay from 45 NMOSD patients for whom more than one titer measurement taken in 6–12 month interval periods was available. The AQP4-IgG titer was measured by a live cell-based assay method, and the serum titer levels between the acute phase and preceding chronic phase were compared. In addition, we evaluated the correlation between the serum titer and relapse frequency while following the clinical course of the enrolled NMOSD patients.

Results: Serum AQP4-IgG titer was not elevated in the acute phase, compared to that of the preceding chronic phase, irrespective of the clinical phenotypes. Moreover, there was no correlation between the titer at onset and relapse frequency in 10 years post-onset or neurological disability at 5 and 10 years after onset. The titer was slightly elevated several months before relapses in about half of the cases, but the change was trivial and may not be applicable for clinical use.

Conclusion: Although evaluating the positivity of serum AQP4-IgG at the onset is necessary, the titer level does not reflect the ongoing disease activity or the following neurological prognosis. Repeated follow-up of titer levels may not be useful for the management of NMOSD patients.

1. Introduction

Neuromyelitis optica spectrum disorder (NMOSD) is an autoimmune neurological disorder affecting the central nervous system (CNS), characterized by the presence of serum anti-aquaporin-4 antibody (AQP4-IgG) [1,2], and by repeated clinical episodes of optic neuritis and myelitis [3]. The diagnostic utility for checking the presence of AQP4-IgG in the serum has been already established. Based on previous research, AQP4-IgG is believed to be one of the primary factors involved in the pathogenesis of NMOSD, possibly affecting the lesion distribution [4,5].

Aquaporins (AQPs) are water channel proteins widely expressed in the animal and plant kingdoms [6,7]. These molecules have six membrane-spanning domains, mainly functioning to facilitate the transportation of water between cells [6,7]. In mammalian cells, they are classified into 13 subtypes, AQP0-AQP12 [7]. AQP4, one subtype, is abundantly expressed in the CNS, especially at astrocyte end-feet that ensheath microcapillary endothelial cells [8–10]. Because of the location of AQP4, peripherally produced AQP4-IgG is believed to have easy access to the antigen when the blood-brain barrier (BBB) is disrupted by factors such as infectious events, leading to the development of brain lesions characteristic of NMOSD [8,10].

Although checking the presence of serum AQP4-IgG is one of the most important diagnostic criteria in NMOSD, the mere presence of AQP4-IgG in the peripheral circulation seems to have no pathological effects on the CNS. At present, the clinical usefulness of serum AQP4-IgG titration for estimating ongoing disease activity or for predicting the neurological prognosis is inconclusive. Evaluation of the serum

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AQP4-IgG titer in each phase of the clinical course is needed to elucidate the clinical utility of measuring and following the antibody titer in NMOSD patients. In this study, by enrolling a number of NMOSD patients who had the serum AQP4-IgG titer measured at regular intervals, we evaluated the clinical significance of documenting serum titer levels in NMOSD patients.

2. Materials and methods

2.1. Patients and study design

A total of 45 patients affected with NMOSD who were positive for the presence of serum AQP4-IgG and had more than two AQP4-IgG titer measurements while on relapse prevention therapy (RPT) with low-dose oral prednisolone were enrolled for this study. All patients were treated at a single university hospital. All NMOSD patients who agreed for repeated AQP4-IgG titration were enrolled for this study.

For each patient, we counted the number of relapses in the 6 months both preceding and following each titration of AQP4-IgG. The timing of each titration was classified into one of the following three groups: the acute phase (defined as < 1 month from the preceding clinical episode), the subacute phase (defined as 1–3 months from the episode), and the chronic phase (defined as > 3 months from the last attack). Measurements of the AQP4-IgG titer in cases followed-up without RPT or measurements taken within 12 months after plasmapheresis were excluded from the analyses.

2.2. Serum AQP4-IgG titration

The serum AQP4-IgG titer was measured with a microscopic live cell-based assay (CBA) method, as previously reported [11,12], using HEK293 cells expressing the human M23-AQP4 protein and an Alexa 488 conjugated secondary antibody (H10120, Thermo Fisher Scientific, *Rockford, IL, USA*) with 1:400 dilution. Fig. 1 shows the positive staining pattern of the M23-AQP4-expressing cells in the CBA. In the human body, AQP4 is expressed as two isoforms - a long isoform with translation initiated at the first methionine (M1-AQP4) and a short isoform with translation initiated at the second methionine (M23-AQP4) [8,10,13]. Both isoforms usually form tetramers, but M23-AQP4 can also assemble into supramolecular aggregates called orthogonal arrays of particles (OAPs). OAPs have a much higher affinity to AQP4-IgG than M1-AQP4 molecules without OAPs [8,10,13]. Therefore, in this study, we conducted the live CBA using M23-AQP4-expressing cells to detect AQP4-IgG. The AQP4-IgG titers were semi-quantitatively evaluated with a two-fold end-point dilution method. For statistical convenience, the antibody titer was represented by the binary logarithm of the titer level, calculated as $log_2(titer)$. AQP4-IgG titers of ≤ 16 were considered seronegative, and their $\log_2(titer)$ were regarded as 4, because changes within such low titer levels would be too large once converted into a logarithmic scale.

First, to elucidate the impact of AQP4-IgG titer in the occurrence of attacks, the serum titer levels were compared between the acute phase and the chronic phase in each patient.

2.3. Chronological relation between AQP4-IgG titer and relapse rate

To elucidate the clinical significance of titration at the onset, we evaluated the correlation between the AQP4-IgG titer level at onset prior to starting treatment for attacks or RPT and the number of relapses in the first 10 years.

Next, we evaluated the relationship between the change in the AQP4-IgG titer level, described as Δ (log_2[*titer*]), and the change in the number of relapses in the preceding or following 6 months, described as Δ (*relapses*).

$$\Delta\,(\log_2[\text{titer}]) = \log_2(\text{titer})_{t(n+1)} - \log_2(\text{titer})_{t(n)}$$

$$\Delta\,(\text{relapses in the following 6 months}) = (\text{relapses in the following 6 months})_{t(n+1)} - (\text{relapses in the following 6 months})_{t(n)}$$

In the above equations, *t(n)* and *t(*n + *1)* are successive tandem time points at which AQP4-IgG titers were obtained during the chronic phase in each patient. The former is the earlier time point and the latter is the later time point within the interval period of 6–12 months. Because the interval period of the selected tandem data was longer than 6 months, the follow up period for number of relapses at each time point did not overlap each other. To minimize the bias based on the preceding treatments for relapses, we excluded the data in the acute or subacute phase (i.e. ≤ 3 month from the preceding episode) in the time series analysis. Also, titers measured within 12 months from plasmapheresis were also excluded from the following analyses.

2.4. Statistical analysis

Comparisons of the variables between two non-paired groups were performed by the Student's *t*-test or Mann-Whitney *U* test. A paired comparison of $log_2(titer)$ between the acute phase and the preceding chronic phase in each of the enrolled patients was performed by the paired *t*-test or Wilcoxon signed-rank test based on the distributional pattern in each group. Correlation between the titer level and number of relapses in the following 6 months was evaluated by the Pearson's R.

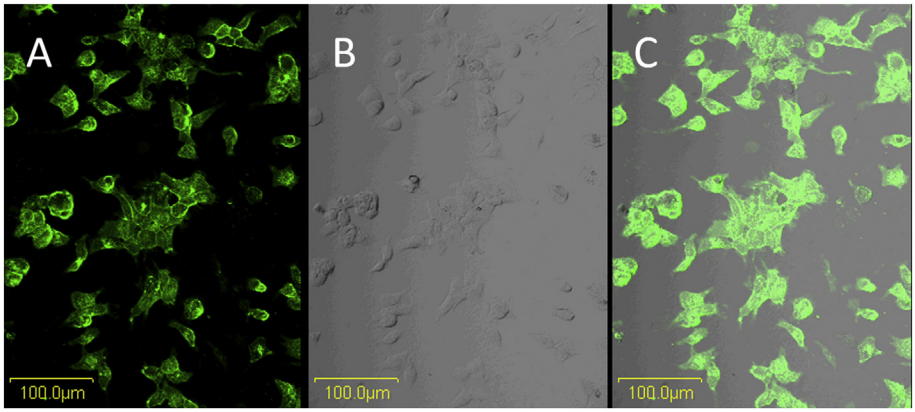

Fig. 1. Positive staining pattern of AQP4 expressing cells in CBA.
(A) HEK293 cells transfected with M23-AQP4 were stained with a serum of NMOSD patient and Alexa 488-conjugated goat anti-human IgG antibody. (B) Bright-field micrograph of the cells. (C) Merge of A and B. The cell surface was stained positive.

Correlation between the titer level at the onset and number of relapses in the first 10 years or the Expanded Disability Status Scale (EDSS) at 5 and 10 years from onset was evaluated by the Spearman's rho, since these clinical variables exhibited a non-normal distribution. A *p*-value < .05 was defined to be statistically significant in this study. Statistical analyses were conducted using either SPSS Statistics Base 22 software (IBM, Armonk, NY, USA) or MATLAB R2015a (MathWorks, Natick, MA, USA).

2.5. Institutional review board

This study was approved by the Institutional Review Board of Tohoku University Hospital (IRB No. 2010589), and written informed consent was obtained from all enrolled patients.

3. Results

3.1. Patient background

Among the 45 enrolled patients, only 1 was male and the rest were female. All the patients were treated with a low oral-dose of prednisolone with (n = 8) or without (n = 36) azathioprine for relapse prevention during the evaluation period. Rituximab was not used during the duration of this study. The age at onset was 41.4 ± 14.1 years [mean ± standard deviation (SD)], and the disease duration at titration was 8.1 ± 9.2 years. AQP4-IgG titers at clinical onset before starting treatments were obtained in 29 patients, with 9 having optic neuritis (ON) and the other 20 having myelitis or medullary lesions.

For the comparison of serum titer between the acute and preceding chronic phases, we collected a total of 23 data pairs from 16 patients. The last data within 12 months before the subsequent relapse was used as the data for the preceding chronic phase. Next, after excluding the acute/subacute phase data, we collected a total of 37 data pairs representative of the chronic phase within a 6–12 month interval from 23 patients.

3.2. Titer in the acute phase before treatments

A total of 23 data pairs from 16 patients of the AQP4-IgG titers in the acute phase of relapses before starting attack treatments (e.g. steroid pulse therapy, plasmapheresis) and in the preceding chronic phase with only oral RPT (e.g. low-dose oral prednisolone) were obtained. The distributions in both phases are shown in Fig. 2A. The titer was not significantly changed in the acute phase compared to that in the preceding chronic phase (p = .56, paired *t*-test). The titer in the acute phase of relapses was not different between relapses with ON or with other phenotypes (i.e. myelitis or medullary lesions).

Meanwhile, the AQP4-IgG titer at the clinical onset (n = 29) was different based on the clinical phenotype of the initial attack. The titer was higher in the patients whose initial attack was with ON than in those with myelitis or medullary lesions (p = .0103, Mann-Whitney *U* test). This difference was still observed even after titer levels were adjusted based on the age of onset.

3.3. Titers during the active period with clustered attack occurrence

Based on the recent knowledge that the attack occurrence in NMOSD forms uneven clustering [14], we also compared the titer levels between those during active phase with clustered attack occurrence (i.e. < 12 months from the last attack) and those during non-active intermittent phase without recent attack occurrence (i.e. ≥12 months from the last attack). As shown in Fig. 2B, the peak level of titer was not significantly different between the active "clustered" phase and non-active intermittent phase (p = .36, Wilcoxon signed-rank test).

3.4. Changes in AQP4-IgG titers after treatments for attacks

Paired data of AQP4-IgG titers before and after the therapeutic interventions for acute attacks, such as intravenous methylprednisolone pulse therapy (IVMP) or plasma exchange (PE), was obtained from 27 attack occasions in 13 patients. The titers taken after treatments for attacks were obtained within one month of administering the therapeutic interventions. Of the 27 therapeutic interventions, 13 were IVMP, 10 were PE, and 4 utilized both IVMP and PE. The changes in serum levels of the AQP4-IgG titer according to the type of therapy are shown in Fig. 3. Both IVMP and PE, regardless of whether they were administered alone or in combination, significantly contributed to decreased levels of the AQP4-IgG titer. For reference, change of serum AQP4-IgG titer between at the onset (acute phase) and after starting low-dose oral prednisolone for relapse prevention (chronic phase) is shown in Fig. 3D. Different from the treatments for acute attacks, low-dose oral prednisolone for relapse prevention did not significantly decrease the serum AQP4-IgG titer even after 1 year (p = .13, ANOVA) or 3 years (p = .49) from starting the medication.

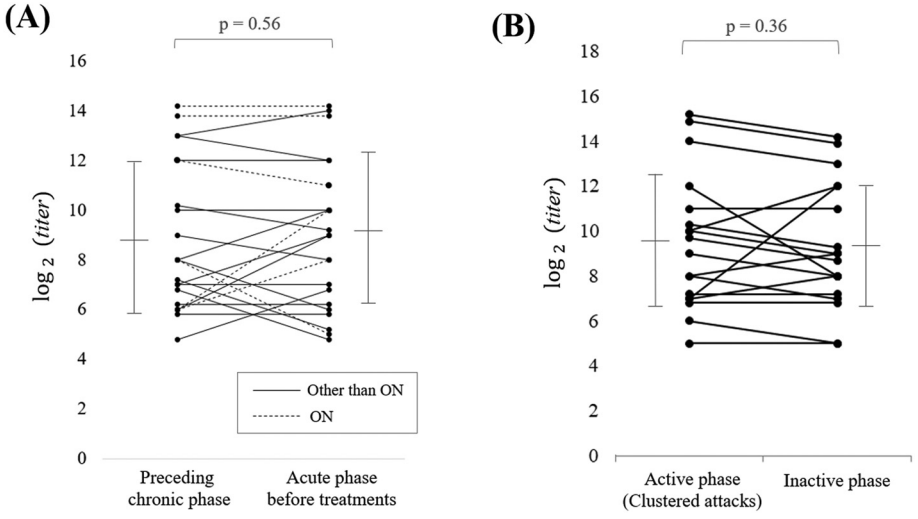

Fig. 2. Clinical usability of serum AQP4-IgG titer.
(A) Serum AQP4-IgG titer in the acute phase was not elevated compared to that in the preceding titer. The bars beside the plots represent mean ± standard deviation. (B) Titer in the active phase with frequent attack occurrence and in the inactive intermittent phase.

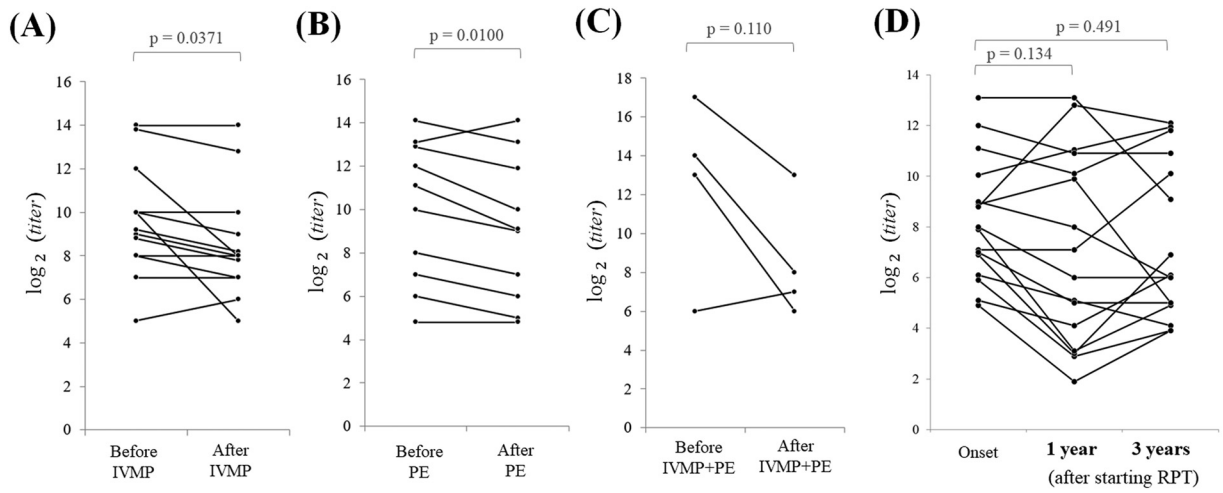

Fig. 3. Changes of AQP4-IgG after treatments for attacks.
(A) Changes of titers with intravenous methylprednisolone pulse therapy (IVMP). (B) Changes of titers with plasma exchange (PE). (C) Changes of titers with both IVMP and PE. Both the IVMP and PE significantly contributed to decrease serum AQP4-IgG level. (D) Low-dose oral prednisolone for relapse prevention did not significantly decrease the titer even after 1 year or 3 years from starting the medication. Abbreviations: IVMP, intravenous methylprednisolone pulse therapy; PE, plasma exchange; RPT, relapse prevention therapy.

3.5. Correlation between AQP4-IgG titer at onset and relapse rate

Among the 29 patients whose serum AQP4-IgG titers were evaluated at the onset before starting treatments for acute attacks or RPT, 26 patients were followed for > 10 years as of May 2019. The scatter plot of the titers at onset and the number of relapses in the first 10 years in each of the 26 patients are shown in Fig. 4. There was no significant correlation between the titers at the onset and the relapse frequency in the following 10 years (Spearman's rho: −0.0086; p = .97).

Also, correlations between the titer at onset and neurological disability, represented by expanded disability status scale (EDSS), at 5 years and 10 years from onset were evaluated. The result of the EDSS at 5 years (Spearman's rho = 0.157, p = .44; n = 26) and at 10 years (Spearman's rho = 0.268, p = .32; n = 16) did not show significant correlation with the AQP4-IgG titer at onset.

3.6. Correlation between changes in AQP4-IgG titer and relapse rate

In evaluation of the correlation between the change in the titer level, described as Δ (log_2[*titer*]), and the change in number of relapses in the 6 months following measurement of the titer level, we observed a significant strong positive correlation between these variables (Pearson's R = 0.547, p < .001). On the contrary, we observed a weak negative correlation with the change in the number of relapses in the 6 months preceding measurement of titers (R = −0.269, p = .11). The correlation matrix, with a 95% confidence interval of the correlation coefficients, is shown in Table 1. To visually confirm the correlation between the change in serum titer and the change in the number of relapse occurrences, a scatter plot of the 37 changes from 23 patients is shown in Fig. 5. Although this would suggest that changes in titer level would predict the relapse frequency, approximately half of the occasions with changed titer levels were not followed by changed relapse frequency.

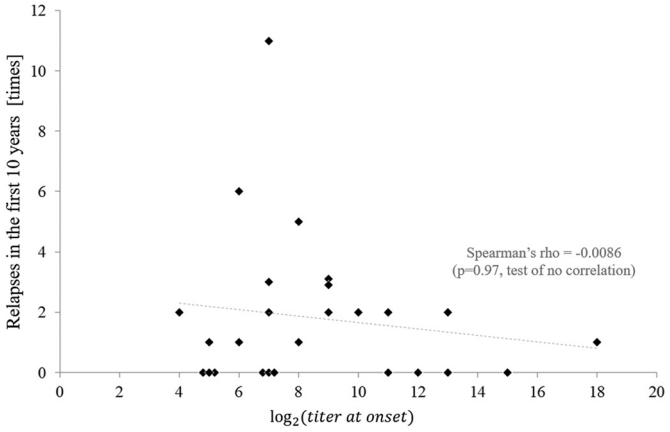

Fig. 4. Impact of AQP4-IgG titer at onset in predicting relapse rate.
Serum AQP4-IgG titer at the onset was not correlated with the relapse frequency in the following 10 years.

4. Discussion

This study showed that the serum AQP4-IgG titer was not elevated in the acute phase but was elevated in the preceding chronic phase. Despite belief that serum titer levels would vary based on the different clinical phenotypes of the initial attack (i.e. ON or myelitis), the titer levels at onset were not predictive of relapse frequency or neurological disability in the first 10 years following the initial attack. Although the titer may have slightly increased several months before the relapses, about half of the occasions with changed titer levels were not followed by changed relapse frequency in the next 6 months, as shown in Fig. 5. Certainly, checking for the presence of serum AQP4-IgG in NMOSD patients is essential for correct diagnosis and the presence of this antibody could be one of the triggers causing attacks; however, the results of this study suggest that other unidentified factors must be identified to predict disease severity and attack frequency.

To reconcile the two apparently conflicting findings that the titer was slightly elevated before the relapse increment but it did not elevated in the acute attacks, it seems to be that we need to suppose some unidentified factors in addition to serum AQP4-IgG that regulate attack occurrence and neurological disability. One of the possibilities may be that the AQP4-IgG level in the cerebrospinal fluid, which was not measured in this study, could be the key factor in regulating attack occurrence, rather than the serum titer level. In some previous reports, AQP4-IgG titer level was thought to be elevated in the acute phase of attacks only in the cerebrospinal fluid, not in the sera [12,15]. Comparing the comprehensive laboratory data between acute attack phase and chronic phase in each patient would be useful in detecting such additional unknown factors that trigger attacks in NMOSD.

Of the enrolled patients, seven patients exhibited seronegative

Table 1
Correlations between the change of antibody titer and change of relapse frequency.

	Δ (log_2[*titer*])	Δ (relapses in the preceding 6 months)	Δ (relapses in the following 6 months)
Δ (log_2[*titer*])	–	−0.269 (−0.545–0.061)	0.547 (0.272–0.740)
Δ (relapses in the preceding 6 months)	p = .11 (n = 37)	–	−0.236 (−0.520–0.096)
Δ (relapses in the following 6 months)	p < .001 (n = 37)	p = .16 (n = 37)	–

The shown correlation coefficients are Pearson's R with its 95% confidence intervals, and the *p*-values are the results of the test of no correlation. Δ: change after an interval period of 6–12 months; Δ (log_2[*titer*]), change in log_2(*titer*) with an interval of 6–12 months; Δ (relapses in the preceding 6 months), change in the number of relapses observed in the preceding 6 months; Δ (relapses in the following 6 months), change in the number of relapses observed in the following 6 months with an interval of 6–12 months.

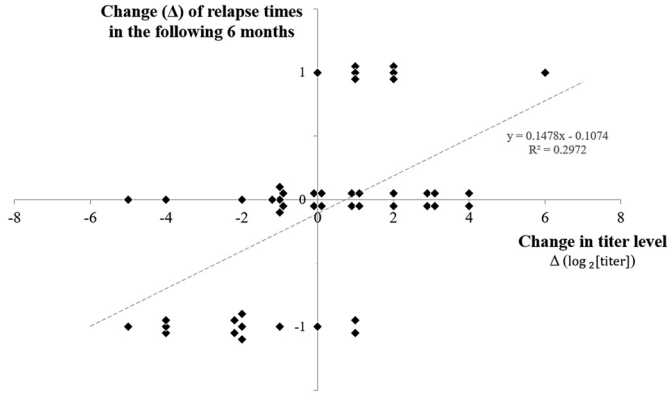

Fig. 5. Correlation between the changes in AQP4-IgG titer and relapse frequency in the following 6 months.
Scatter plot of the changes in serum titer and in the following relapse frequency is shown. Changes in the serum titer, expressed as Δ (log_2[titer]), showed moderate correlation with changes in relapse frequency of the following 6 months. However, about half of the occasions with changed titer level were not followed by changed frequency of relapses.

conversion of the AQP4-IgG titer during clinical course, but two of the seven experienced relapses during steroid tapering more than five years after seronegative conversion. This suggests that even seronegative conversion will not guarantee that the patient is risk-free of future relapses. Certainly, the titer levels decreased after treatments for acute attacks (i.e. IVMP or PE), but the expected rate of reduction was not overly significant. Thus, this information that the titers diminish after treatments for acute attacks may not be enough to determine whether to continue or discontinue these treatments upon clinical attacks.

Because the enrolled patients in this study were entirely comprised of those of Asian origin, further clinical studies with Caucasians and African-American patients are needed to verify the conclusions of this study. Another limitation of this study is that the titer levels were measured using the CBA method, which is generally thought to be less suitable for quantification than the enzyme-linked immunosorbent assay (ELISA) method.

In conclusion, although checking the presence of serum AQP4-IgG is essential for making a correct diagnosis and selecting an appropriate long-term therapeutic strategy, the serum titer levels may not be useful for estimating relapse timing or frequency. Moreover, repeated measurement of the titer may not be useful in the management of NMOSD patients because increases in the titer level have low specificity for increased relapse rate in the following 6 months. Further considerations and research in molecular biology will be needed to identify factors other than AQP4-IgG that reflect attack occurrence or disease activity in NMOSD.

Author contributions

All authors made substantial intellectual contributions to the study, critically reviewed the manuscript, and approved the final version of it. T.A. and T.T. conceived and designed the experiments; T.A., T.T., and I.N. drafted the manuscript; I.N., M. Aoki, and K.F. supervised the whole process of this study.

Declaration of Competing Interest

T. Akaishi and T. Takahashi report no disclosures. I. Nakashima received speaker honoraria and travel funding from Mitsubishi Tanabe Pharma, Biogen Japan, and Novartis Pharmaceuticals and received research support from LSI Medience Corporation. M. Abe, T. Ishii, and M. Aoki report no disclosures. K. Fujihara received speaker honoraria and travel funding from Bayer, Biogen Japan, Eisai, Mitsubishi Tanabe, Novartis, Astellas, Takeda, Asahi Kasei Medical, Daiichi Sankyo, and Nihon Pharmaceutical and received research support from Bayer, Biogen, Asahi Kasei Medical, The Chemo-Sero-Therapeutic Research Institute, Teva, Mitsubishi Tanabe Pharma, Teijin, Chugai, Ono, Nihon Pharmaceutical, and Genzyme.

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