

Anti-aquaporin-4 antibody is involved in the pathogenesis of NMO: a study on antibody titre

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NMO-IgG is a disease-specific autoantibody for neuromyelitis optica (NMO) and its target antigen is aquaporin-4 (AQP4) water channel. Recently, we established a sensitive anti-AQP4 antibody assay using human AQP4-transfected cells, which appeared more sensitive than the original NMO-IgG assay. So far, there has been no large-scale study on anti-AQP4 antibody titre in NMO and related disorders. We tested 148 sera of patients with NMO, high-risk syndrome of NMO, multiple sclerosis (MS), clinically isolated syndrome suggestive of MS and miscellaneous diseases. We analysed the relation of anti-AQP4 antibody titres and clinical and laboratory parameters. The sensitivity of anti-AQP4 antibody assay was 91% (95% CI 79–100) for NMO and 85% (65–100) for high-risk syndrome, and the specificity was 100% (91–100) for NMO and high-risk syndrome, that is, none with the other disorders was positive. Among 21 anti-AQP4 antibody-positive cases whose NMO-IgG were tested, 15 were NMO-IgG-positive and 6 were NMO-IgG-negative. Higher anti-AQP4 antibody titres were associated with complete blindness and extensive or large cerebral lesions on MRI. The lengths of spinal cord lesions on MRI were positively correlated with the titres of anti-AQP4 antibody at the nadir of exacerbations. A few patients who had short (approx. one to two vertebral segments) spinal cord lesions on MRI were also seropositive with low anti-AQP4 antibody titres, but did have other clinical and MRI features of NMO. Anti-AQP4 antibody titres became lower after high-dose methylprednisolone, and a follow-up showed anti-AQP4 antibody titres remained low in relapse-free periods under immunosuppression. Cerebrospinal fluid (CSF)-anti-AQP4 antibody was detected when the serum-antibody titres exceeded 512 \times , at the ratio of 1 (CSF) to 500 (serum). Using a sensitive assay, the results of the present study suggest that NMO and high-risk syndrome may be essentially anti-AQP4 antibody-associated disorders, and that the anti-AQP4 antibody titres have significant clinical and immunological implications in NMO.

Keywords: neuromyelitis optica; NMO-IgG; anti-aquaporin-4 antibody; antibody titre; clinical features

Abbreviations: AQP4 = aquaporin-4; AZT = azathioprine; CIS = clinically isolated syndrome suggestive of MS; CSF = cerebrospinal fluid; HIMP = high-dose intravenous methylprednisolone; MS = multiple sclerosis; NMO = neuromyelitis optica; PE = plasma exchange; PSL = prednisolone; VS = vertebral segments

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Introduction

Neuromyelitis optica (NMO) is a devastating neurological disease characterized by severe optic neuritis and transverse, longitudinally extensive myelitis (Wingerchuk *et al.*, 1999). Recently, its disease-specific serum autoantibody, NMO-IgG, was discovered in an indirect immunofluorescence method (Lennon *et al.*, 2004), and based on a study of the sera of 13 patients with high titres of NMO-IgG, its target antigen was identified as aquaporin-4

(AQP4) water channel protein mainly expressed in the brain and spinal cord (Lennon *et al.*, 2005). NMO-IgG was highly sensitive (58–76%) and specific (94–100%) to NMO, and NMO-IgG seropositive status is now incorporated in the revised diagnostic criteria of NMO (Lennon *et al.*, 2004; Wingerchuk *et al.*, 2006). However, since the substrates of the NMO-IgG detection assay were not human but mouse brain tissues (Lennon *et al.*, 2004), there has been a concern that it might affect its sensitivity and specificity of the

antibody detection. We recently established a highly sensitive assay for anti-human AQP4 antibody using human AQP4-transfected cells as substrates of the indirect immunofluorescence assay (Takahashi *et al.*, 2006). The target antigen of this assay was human-AQP4 molecules and this was strikingly different from the original NMO-IgG assay, which was detected on mouse brain tissues. Eight out of 10 NMO serum samples were positive in that assay, and of those eight anti-AQP4 antibody-positive sera, six were also positive in the original NMO-IgG assay, but the other two were NMO-IgG-negative. Meanwhile, none of the sera from multiple sclerosis (MS) or other neurologic diseases were seropositive (Takahashi *et al.*, 2006). Thus, the new anti-AQP4 antibody assay is expected to have a higher sensitivity than the original NMO-IgG assay.

Here, we expanded the analysis, and tested additional 123 serum samples (148 serum samples in total) of patients with NMO and related disorders for the presence of anti-AQP4 antibody. Then, we analysed the clinical and immunological implications of the anti-AQP4 antibody status and its titres in the cerebrospinal fluid (CSF) as well as the sera in NMO and related conditions.

Materials and methods

Patients, sera and CSF

We analysed 148 clinically ascertained Japanese patients including 25 patients described in our previous report (Takahashi *et al.*, 2006). The patients were diagnosed according to the following criteria. NMO was defined as cases fulfilling all items of 2006 NMO criteria (Wingerchuk *et al.*, 2006) except for NMO-IgG seropositive status. All of these NMO cases ($n=22$) had documented relapses. High-risk syndrome of NMO ($n=13$) was defined as either of the following three. (i) Recurrent optic neuritis without brain lesions. (ii) Myelitis with ≥ 3 vertebral segments (VS) spinal cord lesions with or without brain lesions. (iii) Optic neuritis and/or myelitis without ≥ 3 VS spinal cord lesions with compatible brain lesions for NMO [based on the report by Pittock *et al.* (2006a, b): symmetric diffuse white matter lesions, symmetric diencephalic lesions and symmetric periaqueductal lesions]. MS ($n=53$) was defined as clinically definite MS by Poser's criteria (Poser *et al.*, 1983) except NMO and high-risk syndrome. Clinically isolated syndrome suggestive of MS (CIS) ($n=10$) was defined as cases with a single episode without ≥ 3 VS spinal cord lesions. Miscellaneous diseases ($n=50$) included cerebrovascular disease ($n=5$), neuro-Behçet disease ($n=5$, one with severe myelopathy), Guillain-Barré syndrome ($n=5$),

autoimmune encephalopathy ($n=4$), myasthenia gravis ($n=3$), multifocal motor neuropathy ($n=3$), chronic inflammatory demyelinating polyradiculoneuropathy ($n=3$), spinocerebellar degeneration ($n=3$), amyotrophic lateral sclerosis ($n=3$), Parkinson's disease ($n=2$), spinal canal stenosis with severe myelopathy ($n=2$), ischaemic ophthalmopathy ($n=2$), spinal dural arteriovenous fistula with severe myelopathy ($n=2$), HTLV-1-associated myelopathy ($n=2$), Hashimoto encephalopathy ($n=2$), psychosomatic disorder ($n=2$), systemic lupus erythematosus ($n=1$) and metabolic ophthalmopathy ($n=1$). The demographic data of the main groups are described in Table 1. All specimens (serum and CSF) were obtained from patients seen in 2000–2007 and cryopreserved at -80°C until assayed in the present study in 2006–2007. Anti-AQP4 antibody assay of the specimens was undertaken without knowledge of clinical and laboratory information.

The present study was approved by the institutional review board of the Tohoku University School of Medicine, Sendai, Japan. Patients gave oral consent for testing.

Anti-AQP4 antibody assay

We detected and titrated anti-AQP4 antibody by the method described in our previous report (Takahashi *et al.*, 2006). This technique using HEK-293 transfected with AQP4 was originally reported by Lennon *et al.* (2005). Briefly, human embryonic kidney (HEK-293) cells were stably transfected with the vector with or without AQP4-cDNA, and the specimens were tested by the indirect immunofluorescence method using these two cell lines (with or without AQP4). Specimens were incubated with the cells for 1 h, washed in phosphate buffered saline (PBS), incubated with fluorescein-conjugated goat anti-human IgG (ICN Biomedicals, Aurora, OH, USA) for 30 min and washed in PBS. Then, the cells were fixed in 4% paraformaldehyde and mounted in the aqueous mounting media Permafluor (Beckman Coulter, Marseille, France). Positive samples were titrated in doubling dilutions to ascertain the maximum dilution that yielded positive results, and we basically described the maximum value as the titre of each case in this study. In the detection and the titration assay previously described, we used only cells at 1–10 passages to avoid the influence of repeated passages on the expression of AQP4. We repeatedly titrated the same sera using those cells in a blinded manner and confirmed that the antibody titres were reproducible. In addition, we used sera with known antibody titres for the titration study in every assay and confirmed that the same titres were obtained every time. The cut-off values (minimum dilution of serum or CSF) were $4\times$ in the serum assay and $1\times$ in the CSF assay. Antibody titration was done by two independent investigators.

Table 1 Clinical profile of patients

	Nueromyelitis optica	High-risk syndrome	Multiple sclerosis
Sex: M/F	0/22	0/13	10/43
Age at onset (years) [median (IQR)]	41 (35–53.5)	37 (34–49)	26 (22–31)
Disease duration (years) [median (IQR)]	10 (6.25–14)	5 (2–6)	7 (4–13.5)
Annual relapse rate [median (IQR)]	0.76 (0.57–1.08)	1.0 (0.8–2)	0.69 (0.4–1.0)
Expanded disability status scale [median (IQR)]	6.0 (5.0–6.5)	3.0 (2.5–4.0)	2.0 (2.0–3.5)
Oligoclonal IgG bands (number) [%]	2 (9%)	3 (23%)	30/44 (68%)

Statistical analysis

We compared the demographic and clinical features of the diagnostic categories and subgroups such as anti-AQP4 antibody positive/negative patients or NMO-IgG positive/negative patients. We also compared the anti-AQP4 antibody titres with the clinical features such as permanent complete blindness, the length of spinal cord lesions on MRI and brain MRI findings. In these comparisons, the Mann–Whitney's U test, the Fisher's exact probability test or the regression analysis were used as appropriate. We evaluated the changes of the anti-AQP4 antibody titres with high-dose intravenous methylprednisolone (HIMP) using the Wilcoxon signed-ranks test and the changes of the titres or the number of the relapses in the longitudinal follow-up studies using the Mann–Whitney's U test, the Chi-square for independence test or the Fisher's exact probability test. All of the statistical analyses were considered significant if the *P*-values were less than 0.05. The sensitivity and specificity of anti-AQP4 antibody detection for each diagnostic category were calculated, and 95% CI of these indices were also calculated.

Results

Anti-AQP4 antibody was stably detected by indirect immunofluorescence

Anti-AQP4 antibody was stably detected and titrated as we previously reported (Takahashi *et al.*, 2006). The cell surface was apparently stained by anti-AQP4-antibody-positive sera in a non-permeating condition, strongly suggesting the binding of the serum antibody to the extracellular domain of AQP4 (Fig. 1).

Anti-AQP4 antibody assay was highly sensitive and specific

Twenty of the 22 patients diagnosed with NMO, and 11 of 13 classified as high-risk syndrome were seropositive for anti-AQP4 antibody (Fig. 2). The antibody titres ranged from 16× to 65 536× in NMO, and from 16× to 16 384× in

high-risk syndrome (Fig. 3). None of 53 cases diagnosed as having MS, 10 cases with CIS and 50 cases with miscellaneous disease were seropositive. Thus, the sensitivity of the assay in NMO was 91% (95% CI 79–100), that in high-risk syndrome was 85% (65–100) and the specificity in both NMO and high-risk syndrome was 100% (91–100).

Anti-AQP4 antibody was more frequently detected than NMO-IgG

Of the 31 anti-AQP4 antibody-positive cases, 21 cases had been tested for NMO-IgG. We used the same sera to compare the anti-AQP4 antibody status with the NMO-IgG status. Fifteen cases (71% of 21 cases) were NMO-IgG-positive and six cases were NMO-IgG-negative. Of the 15 NMO-IgG-positive cases, 13 cases were NMO and 2 cases were high-risk syndrome. NMO-IgG titres were available in two cases and their titres were almost identical to those of the anti-AQP4 antibody titres. Of the six NMO-IgG negative cases, two cases were NMO as described in our previous report (Takahashi *et al.*, 2006) and four cases were high-risk syndrome. The clinical features of those four NMO-IgG-negative cases of high-risk syndrome were as follows. One case with 128× of anti-AQP4 antibody had severe recurrent myelitis with ≥ 3 VS spinal cord lesions. The other three cases had optic neuritis and/or myelitis without ≥ 3 VS spinal cord lesions with brain lesions seen in NMO. Of the three cases, one case with 64× of anti-AQP4 antibody had severe optic neuritis, myelitis without ≥ 3 VS spinal cord lesions, bilateral hypothalamic lesions and periaqueductal lesions. Two other cases with 16× of anti-AQP4 antibody had myelitis without ≥ 3 VS spinal cord lesions and periaqueductal lesions with episodes of intractable hiccup, which was also reported to be a characteristic of NMO (Misu *et al.*, 2005a). The comparison of NMO-IgG-positive and negative cases among those who were positive for anti-AQP4 antibody is described in Table 2.

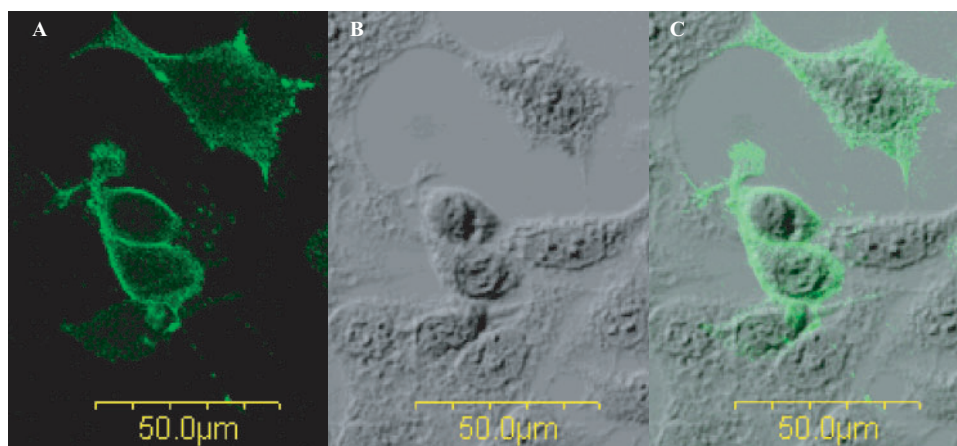


Fig. 1 Indirect immunofluorescence assay using AQP4 expressing cells. (A) HEK 293 cells transfected with AQP4(+) vector (A–C), stained with the serum of a patient with NMO and fluorescein isothiocyanate-conjugated goat anti-human IgG antibody. (B) The cell structures without staining. (C) is a merging of A and B. The surface of three cells was clearly stained positive. The cells surrounding the positively-stained cells are the ones with no expression of AQP4.

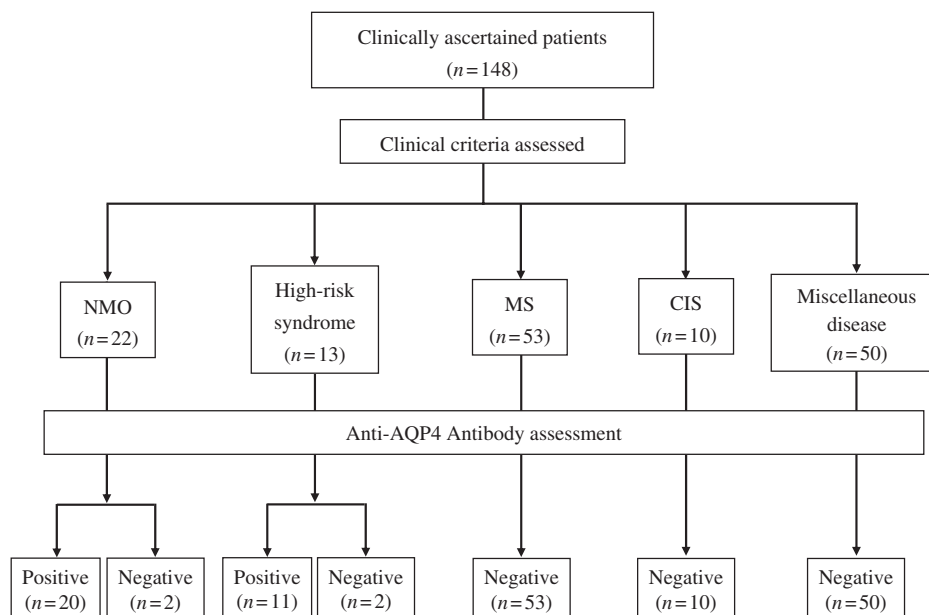


Fig. 2 Main procedures and results in the present study. We used the same format as the one in the report of Lennon and colleagues (Lennon et al., 2004).

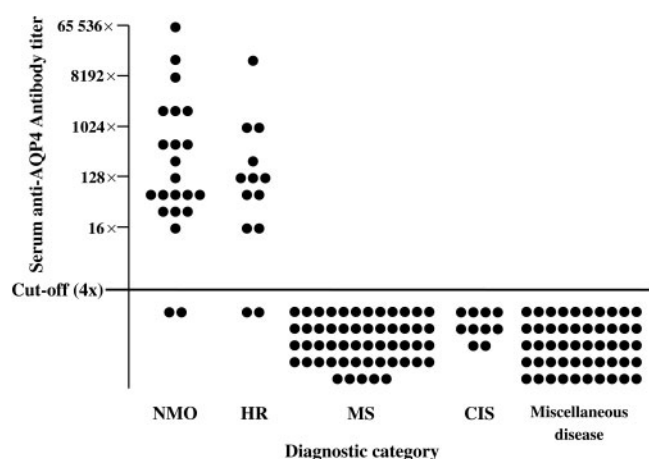


Fig. 3 Serum anti-AQP4 antibody titres in each patient group. We used the same format as the one in the report of Lennon and colleagues (Lennon et al., 2004).

NMO-IgG-positive cases tended to have higher serum anti-AQP4 antibody titres and had a significantly higher frequency of ≥ 3 VS spinal cord lesions. Permanent complete blindness (no light perception) in at least one eye was seen in one-third of the NMO-IgG-positive cases but in none of the NMO-IgG-negative cases.

Serum anti-AQP4 antibody titres were higher in cases with permanent complete blindness or ≥ 3 VS spinal cord lesions on MRI

We compared the serum anti-AQP4 antibody titres with or without permanent complete blindness in at least one eye in the patients with optic neuritis (patients with recurrent

myelitis without optic neuritis were not included). Of the 22 patients with optic neuritis, six patients had blindness (anti-AQP4 antibody titres: median $1536\times$ [range $64\times$ to $65536\times$]) and 15 patients did not have blindness ($64\times$ [$16\times$ to $8192\times$]). The titres were significantly higher ($P=0.0277$) in the patients with permanent complete blindness. We also compared the serum anti-AQP4 antibody titres in patients with ≥ 3 VS spinal cord lesions and those without ≥ 3 VS spinal cord lesions on MRI (the patients with recurrent optic neuritis without myelitis were not included). Of the 29 patients with myelitis, 26 patients had longitudinally extensive lesions (anti-AQP4 antibody titres: median $128\times$ [range $16\times$ to $65536\times$]) and three patients did not have longitudinally extensive lesions (anti-AQP4 antibody titres: one with $64\times$ and two with $16\times$). That is, all of the cases with $128\times$ or more titres of anti-AQP4 antibody had ≥ 3 VS spinal cord lesions and myelitis without ≥ 3 VS spinal cord lesions was seen only in the patients with $64\times$ or lower titres.

A positive correlation was seen between the spinal cord lesion length on MRI and serum anti-AQP4 antibody titres at the nadir of exacerbations

We compared the spinal cord lesion length on MRI and serum anti-AQP4 antibody titres at the nadir of 20 exacerbations in 15 patients. The length of the spinal cord lesions were measured by 0.5 vertebral segment on T2-weighted images. The tested sera for anti-AQP4 antibody titres were obtained within 1 week before or after the evaluated MRI were taken and before any kind of treatments were initiated. A significantly positive

Table 2 Comparison of clinical features between serum-NMO-IgG-positive and -negative cases in the serum-anti-AQP4-positive cases

	Serum anti-AQP4 antibody positive		Group differences [Mann-Whitney's or Fisher's test] P-values
	NMO-IgG positive	NMO-IgG negative	
Sex: M/F	0/15	0/6	
Diagnosis: NMO/High-risk syndrome	13/2	2/4	
Age at onset (years) [median (IQR)]	37 (28–41)	33.5 (32–48)	0.5321
Disease duration (years) [median (IQR)]	10 (6–14.5)	9 (5.25–12.75)	0.5581
Annual relapse rate [median (IQR)]	0.91 (0.6–1.3)	0.8 (0.6–1.4)	0.8762
Expanded disability status scale [median (IQR)]	6.0 (4.5–6.5)	3.5 (3.5–6.0)	0.4475
Serum anti-AQP4 antibody titre [median (IQR)]	256× (64× to 4096×)	64× (32× to 128×)	0.0528
Permanent complete blindness (number) [%]	5 (33%)	0 (0%)	0.1475
Transverse myelitis (number) [%]	13 (87%)	3 (50%)	0.1146
Oligoclonal IgG bands (number) [%]	3 (20%)	1 (17%)	0.6842
Longitudinally extensive spinal cord lesion (number) [%]	15 (100%)	3 (50%)	0.015

correlation ($P < 0.0001$) was seen between the lesion length and the titres (Fig. 4). The two patients with only short spinal cord lesions (1 and 1.5 VS) were cases with high-risk syndrome that had optic neuritis and myelitis with symmetric diencephalic lesions or symmetric periaqueductal lesions, and intractable hiccup and nausea. Also, another patient with a short spinal cord lesion (2 VS) at the time the blood was drawn (Fig. 4) was a case with NMO that developed a longitudinally extensive lesion during another relapse.

Serum anti-AQP4 antibody titres were higher in young adult patients (≤ 45 years) with extensive or large cerebral lesions on MRI

We compared serum anti-AQP4 antibody titres and brain MRI findings in 11 patients who were 45 years old or younger for the purpose of excluding age-related findings such as lacunar infarcts, non-specific periventricular lesions and leukoaraiosis. The anti-AQP4 antibody titres of those patients ranged from 16× to 16 384×. Seven patients with no cerebral lesions and two patients with localized hypothalamic lesions had anti-AQP4 antibody titres of 128× or lower. Meanwhile, three patients with extensive or large cerebral lesions had higher antibody titres (256×, 8192× and 16 384×) (Fig. 5) and the most extensive cerebral lesions were observed in the case with the highest antibody titre (16 384×) (Fig. 5A).

The serum anti-AQP4 antibody titres became lower after high-dose intravenous methylprednisolone (HIMP)

We compared the serum anti-AQP4 antibody titres before and after one or two courses of HIMP in six cases (Fig. 6). The paired sera were obtained shortly before HIMP and

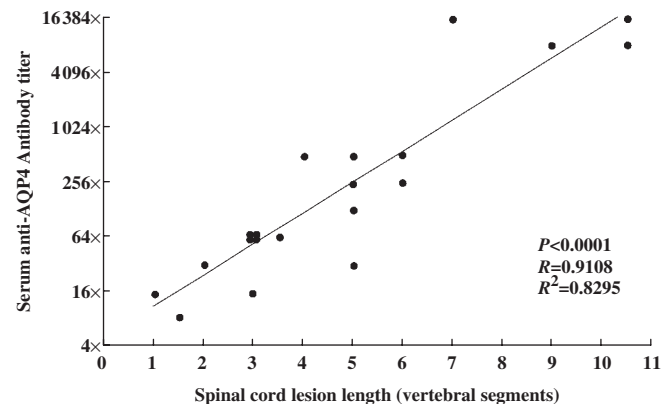


Fig. 4 A positive correlation between the spinal cord lesion length on MRI and the anti-AQP4 antibody titres at the nadir of exacerbations. The spinal cord lesion length was expressed as the number of vertebral segments on T2-weighted images.

1 month after HIMP. The antibody titres significantly decreased after HIMP ($P = 0.0464$).

Anti-AQP4 antibody titres remained low in relapse-free periods under immunosuppressive therapy

There was no significant correlation between the anti-AQP4 antibody titres and the annual relapse rates or EDSS in the patients with NMO and high-risk syndrome. However, we were able to track the time course of serum anti-AQP4 antibody titres and the clinical courses in three cases and found the titres were low in relapse-free periods under immunosuppressive therapy.

Case 1 (Fig. 7A)

A woman with NMO, whose age at onset was 55 years, had an annual relapse rate of 1.25 until May 2003. The serum anti-AQP4 antibody titres were 256× in March 2002

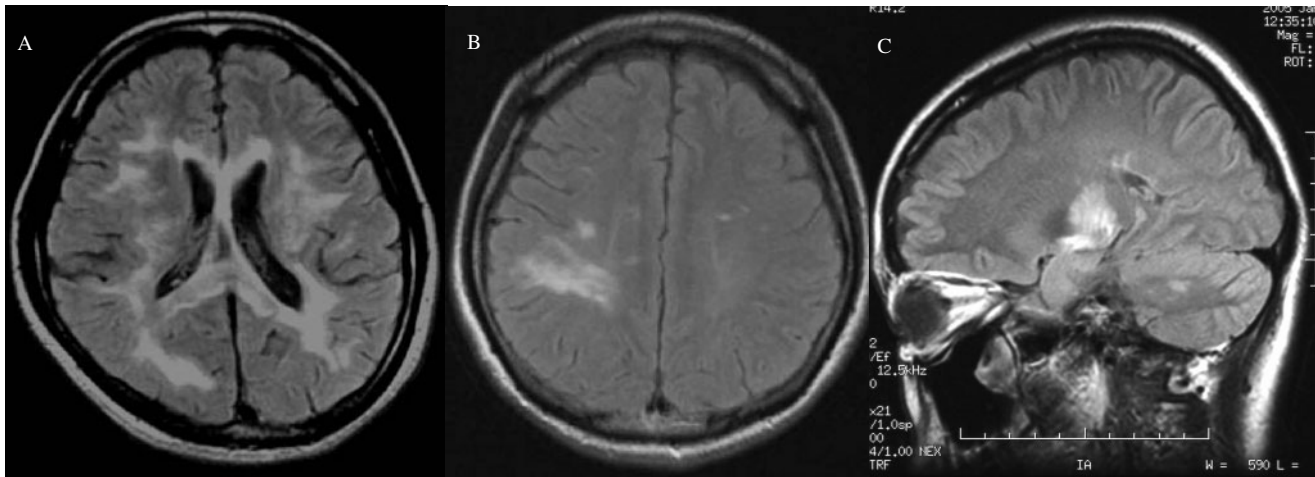


Fig. 5 Extensive or large cerebral lesions on MRI in anti-AQP4 antibody-positive patients aged 45 years or younger. (A) Patient with 16 534 \times titres of anti-AQP4 antibody, fluid attenuated inversion recovery (FLAIR) axial. (B) Patient with 8192 \times titres of anti-AQP4 antibody, FLAIR axial. (C) Patient with 256 \times titres of anti-AQP4 antibody FLAIR sagittal.

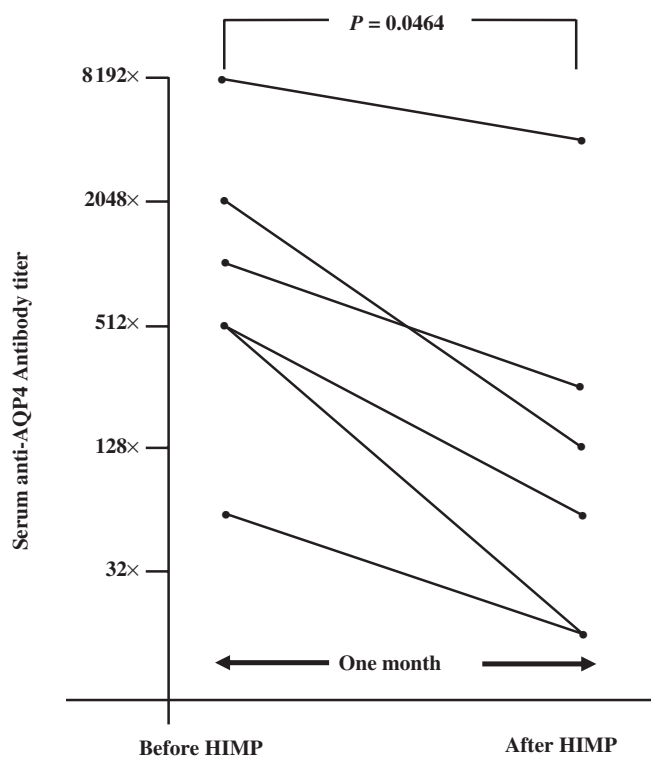


Fig. 6 Comparison of serum-anti-AQP4 antibody titres before and after high-dose intravenous methylprednisolone (HIMP) therapy. The paired sera were obtained shortly before HIMP and 1 month after HIMP. The antibody titres significantly decreased after HIMP ($P = 0.0464$).

and in August 2003, 512 \times in November 2002 and in May 2003. She received two courses of HIMP therapy in May 2003, and the serum was obtained 1 month after HIMP, in June 2003. At that point, the serum anti-AQP4 antibody titre was 16 \times . In June 2003, daily oral prednisolone (PSL) (5–10 mg/day) and azathioprine (AZT) (50 mg/day)

were started and no relapse has been seen up to the present. The serum anti-AQP4 antibody titre was 8 \times in April 2006, and in October and December 2006. The significant decrease has been observed in the anti-AQP4 antibody titres ($P = 0.0165$) and the number of relapses ($P = 0.0278$) since June 2003.

Case 2 (Fig 7B)

A woman with high-risk syndrome, whose age at onset was 37 years, had an annual relapse rate of 1.0 until December 2005. The serum anti-AQP4 antibody titres were 4096 \times in October 2004 and 8192 \times in December 2005. She received two courses of HIMP in December 2005 and four sessions of plasma exchange (PE) in January 2006, but those treatments were not very effective. Then, the fifth exacerbation occurred in February 2006. At that point, the serum anti-AQP4 antibody titre was 16 384 \times . In February 2006, daily oral PSL (5–10 mg/day) and AZT (100 mg/day) were started and no relapse has been seen up to the present. The serum anti-AQP4 antibody titre was 256 \times in August, October, November 2006 and January 2007. The significant decrease has been observed in the anti-AQP4 antibody titres ($P = 0.0098$) after February 2006. No relapse has been seen since February 2006.

Case 3 (Fig 7C)

A woman with high-risk syndrome, whose age at onset was 31 years, had an annual relapse rate of 2.5 until January 2005. The serum anti-AQP4 antibody titres were 256 \times in April, July and October 2004 and in January 2005. In January 2005, daily oral PSL (10 mg/day) was started and no relapse has been seen for over a year. However, 2 weeks after the discontinuation of daily PSL, the sixth exacerbation occurred in November 2005. At that point, the serum anti-AQP4 antibody titre was 128 \times . Daily PSL was

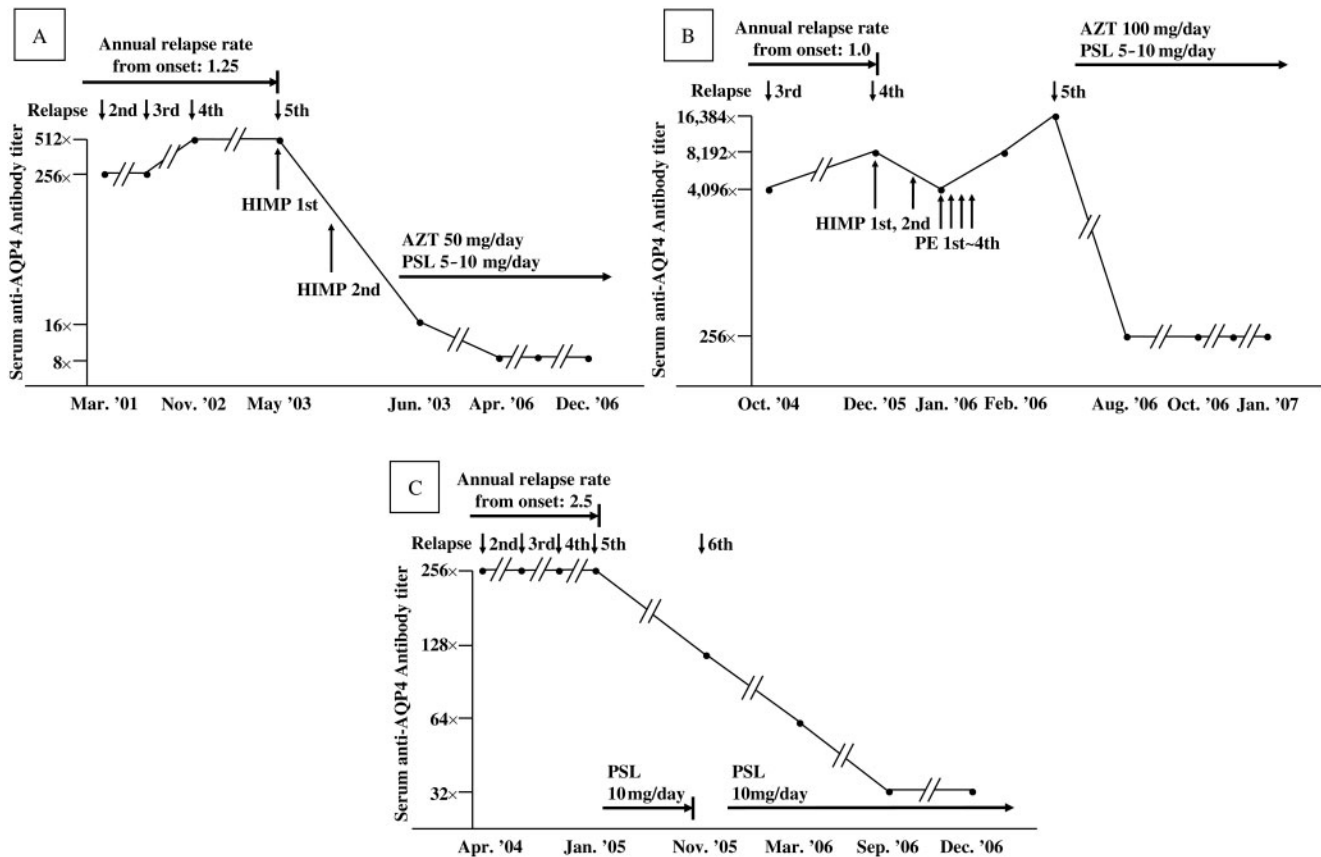


Fig. 7 The time course of serum-anti-AQP4 antibody titres: (A) Case 1, (B) Case 2 and (C) Case 3. PSL = prednisolone; AZT = azathioprine; HIMP = high-dose intravenous methylprednisolone; PE = plasma exchange.

resumed and no relapse has been seen up until the present. The serum anti-AQP4 antibody titres were 64 \times in March 2006 and 32 \times in September and November 2006. The significant decrease was observed in the anti-AQP4 antibody titres ($P=0.0132$) and the number of relapses ($P=0.0427$) since February 2005.

Anti-AQP4 antibody titres were consistently higher in serum than in CSF

Of the 31 serum anti-AQP4 antibody positive cases, 20 cases were examined for the antibody in CSF. Eight cases were positive and 12 cases were negative. Anti-AQP4 antibody was consistently positive in CSF when the serum anti-AQP4 antibody titres exceeded 512 \times and their titres were almost proportional to the serum anti-AQP4 antibody titres at the ratio of 1 (CSF) to 500 (serum). The titres ranged from 1 \times to 128 \times . Cases with 256 \times or lower of serum anti-AQP4 antibody titres were all negative for CSF anti-AQP4 antibody.

Discussion

Using a highly sensitive assay of anti-AQP4 antibody we developed recently (Takahashi *et al.*, 2006), the present study revealed the clinical and immunological implications

of the autoantibody titre in NMO. First of all, we demonstrated that anti-AQP4 antibody is exclusively detected in NMO and high-risk syndrome, and found that the majorities of those two patient groups were positive for autoantibody, strongly suggesting that anti-AQP4 antibody is closely associated with NMO and the high-risk syndrome.

As we showed in our recent report (Takahashi *et al.*, 2006), our anti-AQP4 antibody assay was more sensitive than the original NMO-IgG assay. In that study, all six NMO-IgG-positive patients were seropositive in our anti-AQP4 antibody assay, and there were two NMO-IgG-negative patients who were found to be anti-AQP4 antibody-positive (Takahashi *et al.*, 2006). Both of the patients had definite NMO and one of them had a relatively high titre (2048 \times) of anti-AQP4 antibody. In the present series, we found four additional anti-AQP4 antibody-positive patients with high-risk syndrome who were previously judged NMO-IgG-negative. As a result, the sensitivities of our anti-AQP4 antibody assay were as high as 91% in NMO and 85% in high-risk syndrome, and the specificity in NMO and high-risk syndrome was 100%. The discrepancy of the autoantibody detection between these two assays is probably related to two factors: detection sensitivity and species-specific AQP4 protein sequences.

(i) As compared with the NMO-IgG assay which requires a 60× initial serum dilution and pre-absorption of serum with liver powder (Lennon *et al.*, 2004), our assay needs only a 4× initial serum dilution and no pre-conditioning of the serum. The anti-AQP4 antibody titres were roughly proportional to the NMO-IgG titres in the present study, and thus the low (4× to 32×) anti-AQP4 antibody titres in our assay could be scored negative in the NMO-IgG assay.

(ii) The NMO-IgG assay uses mouse brain slices (Lennon *et al.*, 2004), but we used human AQP4 in our assay. Although human AQP4 is highly homologous to mouse AQP4 (95%), amino acid sequences in the extracellular domains of the water channel protein are different between the two species. The difference may be crucially important since anti-AQP4 antibodies in the patients bound to the surface of AQP4-transfected cells in a non-permeating condition in our study. In fact, the anti-AQP4 antibody titres in some of the patients who were judged negative for NMO-IgG were not low (64× to 2048×). Detailed epitope analyses of anti-AQP4 antibody should be done to address this issue.

In the present study, the anti-AQP4 antibody titres were higher in the cases with permanent complete blindness, longitudinally extensive (≥ 3 VS) myelitis and extensive or large cerebral lesions. In addition, the lengths of the spinal cord lesions were positively correlated with the titres of anti-AQP4 antibody at the nadir of exacerbations. In that analysis, we found that three patients with short (1–2 VS) spinal cord lesions were also positive for anti-AQP4 antibody. The antibody titres in those patients were low, but they had other clinical and MRI features of NMO as described in ‘Results’. The results not only confirmed our recent study using the NMO-IgG assay (Nakashima *et al.*, 2006), but also underscore the significance of anti-AQP4 antibody titres in the development of the unique and severe clinical manifestations of NMO. The clinical and immunopathogenetic importance of the anti-AQP4 antibody titres in NMO was also apparent in the analyses of the therapeutic effects. The anti-AQP4 antibody titres became lower after HIMP, and in the patients whose anti-AQP4 antibody titres were followed longitudinally, there was a consistent tendency that the patients had relapses when the autoantibody titres were higher, but were relapse-free when the titres remained low with daily PSL alone or in combination with AZT. Moreover, in Case 2 (Fig. 7B), despite four plasma exchanges to treat the fourth relapse, anti-AQP4 antibody titre continued to rise and another relapse occurred later. Sustained production of the antibody was probably responsible for the rise of the titre, although we could not measure the antibody titre immediately after plasma exchange due to a lack of the serum at that point. These observations suggest that the anti-AQP4 antibody titres could be related to the disease activity of NMO or the risk of relapse, although various other antibody levels may also be reduced by the therapies. The immunosuppressive therapies to keep

anti-AQP4 antibody titres low could contribute to maintaining remission of this devastating disease in individual patients, although there may not be an absolute threshold of antibody titres for relapses that is applicable to all patients. Longitudinal follow-ups of anti-AQP4 antibody titres in untreated patients also need to be done to determine the fluctuations of antibody titres in the natural course of NMO.

All the aforementioned findings were reflected by anti-AQP4 antibody titres in the serum. Then, what is the significance of anti-AQP4 antibody in the central nervous system? To address this issue, we titrated anti-AQP4 antibody in CSF, and found that CSF-anti-AQP4 antibody was consistently positive when the serum-anti-AQP4 antibody titres exceeded 512×. Moreover, the CSF-anti-AQP4 antibody titres were nearly proportional to the serum-antibody titres at the ratio of 1 (CSF) to 500 (serum). Conversely, cases of NMO and high-risk syndrome which had 256× or less of serum anti-AQP4 antibody titres were all negative for CSF-anti-AQP4 antibody. This means that there is no intrathecal production of anti-AQP4 antibody in NMO, which is in striking contrast to the intrathecal production of immunoglobulins, such as positive oligoclonal IgG bands and high IgG index, in MS.

It is a matter in question how anti-AQP4 antibody is involved in the pathogenesis of NMO. AQP4 is mainly expressed in the periventricular and periaqueductal areas of the brain, and in the gray matter of the spinal cord (Jung *et al.*, 1994; Oshio *et al.*, 2004), and more specifically is concentrated at the astrocytic foot processes which line the outside of the blood–brain barrier (Vizuet *et al.*, 1999). Consistent with the distribution of AQP4, the brain lesions of NMO were reported to be localized in the periventricular and periaqueductal areas (Misu *et al.*, 2005a; Nakashima *et al.*, 2006; Pittock *et al.*, 2006a, b), and myelitis in NMO often involves the central gray matter. In the perivascular areas of those NMO lesions, immunoglobulins and activated complements were deposited (Lucchinetti *et al.*, 2002; Misu *et al.*, 2005b). We and others most recently found the loss of AQP4 in the active perivascular lesions of NMO (Misu *et al.*, 2006, 2007; Roemer *et al.*, 2007). Since the blood–brain barrier is often disrupted in NMO (Bergamaschi, 2003), it can allow the permeation of anti-AQP4 antibodies through the blood–brain barrier into the central nervous tissues to bind to the extracellular domains of AQP4 expressed on astrocytic foot processes. Such antigen–antibody interaction along with complements and immune cells may cause the loss of AQP4 immunoreactivity and eventually damage astrocytes. As shown in our study, the higher the serum-anti-AQP4 antibody titres, the more CSF-anti-AQP4 antibodies there were in the central nervous tissue, possibly causing such pathogenic events. All these findings stress the therapeutic benefits of removing anti-AQP4 antibodies from the circulating blood by appropriate plasma exchange therapy or by lowering the antibody titres with immunosuppressants.

Conclusion

The results of the present study suggest that anti-AQP4 antibody has significant clinical and immunological implications in NMO, and these findings were unobtainable without anti-AQP4 antibody titration by our highly sensitive assay. Subjects for further studies may include (i) analyses of the therapeutic significance of anti-AQP4 antibody titres in a large number of cases to determine whether there is any threshold level of antibody titres in individual cases that can be related to relapse and (ii) careful follow-ups of anti-AQP4 antibody-negative cases with NMO which comprised about 10% of our series. However, our anti-AQP4 antibody assay will be particularly useful to classify cases with intermediate or overlapping features of NMO and MS in Asian populations in whom such cases are relatively common.

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