fourth of CMS patients [2,17–20], reflecting the severe spinal cord damage commonly seen in Asians. In addition, diffused spinal cord lesions were found in 13% of individuals in a Western MS series published recently [21]. These observations render delineation between MS and NMO on the basis of MRI findings to be difficult, especially in Asians.

We previously reported that, according to multiple logistic analyses, only relapse rates, but neither the presence of LESCLs nor EDSS scores, were associated with the presence of anti-AQP4 antibody [12]. Nakashima et al. [11] reported that only severe visual impairment was significantly associated with the presence of NMO-IgG. On the other hand, NMO-IgG was detected in 9% of Western MS patients [8] and 15% of Japanese classical MS patients [11], whereas 30 to 50% of NMO patients or OSMS patients with LESCLs are consistently negative for NMO-IgG/anti-AQP4 antibody [8,11,12,22]. Thus the in vivo role of NMO-IgG or anti-AQP4 antibodies in NMO lesion formation is currently uncertain. Therefore, it is critical to disclose which clinical parameters are associated with NMO-IgG/anti-AQP4 antibody in order to clarify the in vivo role of these antibodies. Multimodality-evoked potentials, including visual-evoked potentials (VEPs), somatosensory-evoked potentials (SEPs) and motor-evoked potentials (MEPs), are useful tools for detecting both clinically overt and silent lesions in the optic nerves and spinal cord. Therefore, in the present study, we first aimed to clarify whether the presence of anti-AQP4 antibody is associated with any abnormalities in evoked potentials (EPs) in a series of MS patients with and without anti-AQP4 antibody. Second, we studied the relationships of the presence of MRI lesions in the optic nerve pathway and the spinal cord with both EP abnormalities and anti-AQP4 antibody status. Finally, we performed multiple logistic analyses to disclose possible factors contributing to the development of severe visual, sensory and motor impairment.

2. Patients and methods

2.1. Patients

One hundred and eleven consecutive patients who were diagnosed with clinically definite MS according to the criteria of Poser et al. [23] at the MS clinic in the Department of Neurology, Kyushu University Hospital, and subjected to both multimodality-evoked potential study and anti-AQP4 antibody assay, during the period 1987–2007, were enrolled in the present study. All patients underwent a thorough neurological examination and routine laboratory tests. All were followed-up and clinically evaluated at regular intervals in the MS clinic. Their medical and EP records were analyzed retrospectively in the present study. None of the patients were seropositive for human T-cell leukemia virus type I. All patients had a relapsing–remitting or relapsing-progressive course, and no patients with primary progressive MS were included in the present study. Patients with monophasic NMO without subsequent relapse were also excluded to avoid including patients with acute disseminated encephalomyelitis.

The disability status of the patients was scored according to the Expanded Disability Status Scale (EDSS) of Kurtzke [24]. Visual acuity was evaluated as: 0, normal; −1, mild visual impairment; −2, finger counting; −3, light perception; −4, total blindness. Muscle power was evaluated as; 0, normal; −1, mild weakness; −2, moderate weakness, −3, severe weakness; −4, complete paralysis. Superficial sensation was evaluated as: 10, normal; 9 to 1, mild to severe; 0, loss while vibration and position sensation were evaluated as: 0, normal; −1, mild impairment; −2, moderate; −3, severe; −4, loss.

2.2. Anti-AQP4 antibody assay

Anti-AQP4 antibody was assayed by an immunofluorescence method described previously [12]. Briefly, a full-length cDNA encoding human AQP4 (AQP4 transcript variant a; GenBank accession number NM_001650) was amplified from a cDNA library generated from commercially obtained human spinal cord mRNAs (Clontech, Mountain View, CA, USA). The PCR product was cloned into the pDONR221 vector (Invitrogen, Carlsbad, CA, USA), and its sequence was confirmed. After sequencing, the AQP4 cDNA was transferred to the pcDNA-DEST53 expression vector (Invitrogen). Human embryonic kidney HEK-293 cells maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum were seeded at 10,000 cells/well onto 8-well chamber slides (Becton Dickinson, Franklin Lakes, NJ, USA) 24 h before transfection. The cells were transfected with 100 ng/well of the GFP-AQP4 fusion protein expression vector using the FuGENE6 Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. At 48 h after transfection, the cells were initially incubated with the human sample serum diluted 1:4 with DMEM for 1 h at 37.0 °C to avoid permeabilization during fixation, washed in phosphate-buffered saline and visualized with an Alexa 594-conjugated goat anti-human IgG antibody (Invitrogen). Fluorescence was observed using a confocal laser-scanning microscope (Flu FV300; Olympus Optical Co., Tokyo, Japan). With the examiners blinded to the origin of the specimens, the anti-AQP4 antibody assay was carried out at least twice for each sample, and those that gave a positive result twice were deemed to be positive.

2.3. Evoked potentials

A checkerboard pattern was back-projected onto a translucent screen. Stimulation was phase reversed at 1 Hz. The stimulating field subtended 8 deg in diameter and the check size was 30 min of arc. The mean luminance was 180 cd/m^2 with a contrast level of 90%. Subjects were instructed to fixate their eyes at the center of the stimulus field at a viewing distance of 100 cm. Monocular full visual fields were stimulated. VEPs were recorded from an electrode placed at Oz with a reference at Fz (International 10–20 system). A total of 100 responses were averaged and repeated at least twice to establish reproducibility. The latency of the major positive peak (P100) was measured [25].

SEPs were obtained by stimulating the median nerve at the wrist and the posterior tibial nerve at the ankle with frequencies of 5 Hz and 2 Hz, respectively. Recording electrodes for upper limbs were placed over Erb's point, the seventh cervical vertebra, and C3′ or C4′ over the somatosensory cortex. For the lower limbs, the electrodes were placed over the 12th thoracic vertebra and Cz′. Fz was used as the reference for all electrodes. A total of 500 responses for the upper limbs and 350 responses for the lower limbs were averaged and repeated at least twice to establish reproducibility. The peak latencies of the responses were measured: N9 (Erb), N13 (C7) and N20 (sensory cortex) for median nerve SEPs and N20 (Th12) and P37 (sensory cortex) for tibial nerve SEPs. The central sensory conduction time (CSCT) was calculated as N20-N13 for the upper limbs and P37-N20 for the lower limbs [26].

MEPs were obtained from the abductor pollicis brevis for the upper limbs and the abductor hallucis for the lower limbs. Magnetic stimuli were applied to the motor cortex and the seventh cervical vertebra (C7) using a figure-of-eight-shaped coil for the upper extremities, while a double-cone coil was used to stimulate the motor cortex for the lower extremities, and the fourth lumbar root (L4) was elicited by the figure-of-eight-shaped coil. We assessed MEP latencies, and the central motor conduction time (CMCT) was calculated as the difference between cortical motor latency and peripheral motor latency [26].

The upper normal limits (mean + 3 SD, ms) of EPs in our laboratory were as follows; VEP P100, 121.0; CSCT of the median nerve SEP, 7.33; CSCT of the posterior tibial nerve SEP, 21.83; CMCT of the upper limb MEP, 10.67; CMCT of the lower limb MEP, 21.04.

Table 1
Demographic features of MS patients in the present study.

	Anti-AQP4 antibody-positive patients ($n=18$)	Anti-AQP4 antibody-negative patients ($n=93$)
Male/female	0/18*	32/61
Age at onset (years)[a]	39.3 ± 14.5*	31.5 ± 12.7
Disease duration (years)[a]	12.6 ± 7.0	11.0 ± 9.2
Annualized relapse rate[a]	1.1 ± 0.7*	0.7 ± 0.6
LESCLs on MRI	13/18 (72.2%)*	36/93 (38.7%)
Optic nerve lesions on MRI[b]	7/34 (20.6%)*	10/162 (6.2%)
EDSS scores at the last examination[a]	5.0 ± 2.5	4.0 ± 2.8

AQP4 = aquaporin-4; EDSS = Expanded Disability Status Scale of Kurtzke; LESCLs = longitudinally extensive spinal cord lesions extending three or more vertebral segments.
*$p<0.05$ in comparison between patients with and without anti-AQP4 antibody.
[a] Mean ± SD.
[b] Each eye (each optic nerve) was examined.

Recording results were divided into three groups according to P100 latency, CSCT and CMCT: the normal group had normal latency, the unevoked group had a lack of cortical responses, at least on one side of the eye or body, and the delayed group had prolonged latency on at least one side.

2.4. Magnetic resonance imaging

All MRI studies were performed using Magnetom Vision and Symphony 1.5-T units (Siemens Medical Systems, Erlangen, Germany), as previously described [12]. We obtained 98 MRI samples from patients who underwent VEP study, 81 from those who were subjected to SEP study, and 86 from those who underwent MEP study. Lesions visible on T2 and FLAIR images were counted.

2.5. Statistical analyses

The frequencies of given observations between anti-AQP4 antibody-positive and -negative patients were compared by Fisher's exact test. Values not normally distributed (age, duration, rate, or scoring) were compared by the Mann–Whitney U test. When multiple comparisons were made, uncorrected p values were corrected by the number of comparisons to calculated corrected p values (Bonferroni–Dunn's correction). Multiple logistic analyses were performed to assess possible factors contributing to development of severe visual impairment (finger counting or worse), severe muscle weakness (moderate weakness or worse) in lower limbs and severe sensory impairment (moderate or more severe position sense impairment) in lower limbs. The variables studied were gender, age at onset, disease duration from onset to time of study, anti-AQP4 antibody status, presence or absence of MRI lesions in the optic nerve pathway or spinal cord, unevoked EP records, corticosteroid treatment at study, plasma exchange at study, and interferon (IFN) beta therapy at time of study. In all assays, statistical significance was set at $p<0.05$.

3. Results

3.1. Demographic features

The demographic features of the patients are summarized in Table 1. Patients with anti-AQP4 antibody showed a significantly higher frequency of females ($p=0.001$), higher age at onset ($p=0.030$), higher annual relapse rates ($p=0.006$), and higher frequency of LESCLs ($p=0.011$) and optic nerve lesions ($p=0.0137$) compared with those without anti-AQP4 antibody, while disease duration was similar between the two groups. The latest EDSS scores were higher in patients with anti-AQP4 antibody than those without the antibody, but the differences were not significant.

3.2. Visual function and VEP findings

The visual functions and VEP findings of the patients are summarized in Table 2. Past and present history of visual impairment at the time of VEP study and the frequency of patients with severe visual impairment (finger counting or worse) were significantly higher among patients with anti-AQP4 antibody than those without the antibody ($p=0.048$ and $p=0.001$, respectively). The visual acuity of anti-AQP4 antibody-positive patients was significantly worse than that of those without the antibody ($p=0.001$). The frequencies of optic atrophy and/or temporal pallor were also higher in anti-AQP4 antibody-positive patients than anti-AQP4 antibody-negative patients, but the difference was not significant. Frequencies of immunotherapies, such as IFN beta, corticosteroid, and plasma exchange, at the time of study, were not significantly different between anti-AQP4 antibody-positive patients and anti-AQP4 antibody-negative patients. P100 was not elicited in 11 of the 17 (64.7%) patients with anti-AQP4 antibody and 20 of the 84 (23.8%) patients without the antibody, while P100 was delayed in one (5.9%) patient with anti-AQP4 antibody and 28 (33.3%) patients without the antibody. As a result, significantly more patients with anti-AQP4 antibody showed a lack of the P100 component than did patients without the antibody ($p=0.003$), whereas the frequency of delayed P100 latency was significantly higher in the latter group than in the former ($p=0.021$). VEP results from each eye were also compared. The frequency of eyes showing a lack of the P100 component was significantly higher among patients with the anti-AQP4 antibody (38.2%) than among those without the antibody (15.1%) ($p=0.003$), whereas the frequency of delayed P100 latency was significantly higher in the latter group (30.1%) than in the former (11.8%) ($p=0.033$) (Fig. 1). VEP findings in the remission phase also showed essentially the same results. The frequency of patients in remission showing a lack of the P100 component was significantly higher among anti-AQP4 antibody-positive patients (69.2%) than among those

Table 2
Visual function and VEP findings in MS patients with and without anti-AQP4 antibody.

		Anti-AQP4 antibody-positive patients ($n=17$)	Anti-AQP4 antibody-negative patients ($n=84$)
Age at time of VEP study (years)[a]		44.7 ± 14.4	39.2 ± 11.9
Age at onset (years)[a]		39.3 ± 14.5*	31.5 ± 12.7
Time elapsed from disease onset to VEP study (years)[a]		6.6 ± 6.8	7.1 ± 8.3
Past and present history of visual impairment at time of VEP study		15/17 (88.2%)*	51/84 (60.7%)
Severe visual impairment (finger counting or worse)		9/17 (52.9%)*	11/84 (13.1%)
Visual acuity[ab]		−1.8 ± 1.6*	−0.6 ± 0.9
Optic atrophy/temporal pallor		8/17 (47.1%)	22/84 (26.2%)
Remission phase percentage at VEP study		13/17 (76.5%)	44/84 (52.4%)
IFN beta therapy at time of VEP study		5/17 (29.4%)	17/84 (20.2%)
Corticosteroid therapy at time of VEP study		14//17 (82.4%)	54/84 (64.3%)
Plasma exchange at time of VEP study		0/17 (0.0%)	3/84 (3.6%)
VEP (P 100)	Unevoked	11/17* (64.7%)	20/84 (23.8%)
	Delayed	1/17* (5.9%)	28/84 (33.3%)
	Normal	5/17 (29.4%)	36/84 (42.9%)

AQP4 = aquaporin-4; IFNβ = interferon β; VEP = visual-evoked potentials.
*$p<0.05$ in comparison between patients with and without anti-AQP4 antibody.
[a] Mean ± SD.
[b] Visual acuity was scored as follows: 0, normal; −1, mild vision impairment; −2, finger counting; −3, light perception; −4, total blindness.

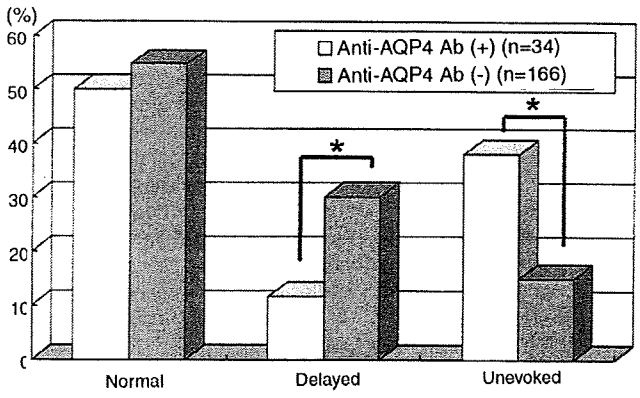

Fig. 1. Results of the VEP study in each eye. *$p<0.05$ in comparison between anti-AQP4 antibody-positive and -negative eyes.

without the antibody (33.3%) ($p=0.028$), whereas the frequency of delayed P100 latency was higher in the latter group (33.3%) than in the former (7.7%), but without statistical significance ($p=0.087$).

3.3. Sensory function and SEP findings

The sensory functions and SEP findings of our patients are summarized in Table 3. Age at SEP examination was higher in anti-AQP4 antibody-positive patients than in anti-AQP4 antibody-negative patients ($p=0.020$). Sensory impairment in upper and lower limbs was not different significantly between patients with and without anti-AQP4 antibody who were subjected to SEP studies. Frequencies of immunotherapies, such as IFN beta, corticosteroid, and plasma exchange, at the time of study, were not significantly different between anti-AQP4 antibody-positive patients and anti-AQP4 antibody-negative patients. A lack of an evoked response in the median nerve SEPs was observed in none of 12 patients with anti-AQP4 antibody and 10 of 60 (16.7%) patients without anti-AQP4 antibody, while a delayed CSCT was found in two of 12 (16.7%) patients with anti-AQP4 antibody and eight of 60 (13.3%) patients without anti-AQP4 antibody. Regarding posterior tibial nerve SEPs, one of 12 (8.3%) patients with anti-AQP4 antibody and 11 of 60 (18.3%) patients without the antibody showed a lack of evoked responses, while two of 12 (16.7%) patients with anti-AQP4 antibody and 16 of 60 (26.7%) patients without the antibody showed delayed CSCTs. There was no significant difference in the frequencies of abnormal findings between anti-AQP4 antibody-positive and -negative patients.

Table 3
Sensory functions and SEP findings of MS patients with and without anti-AQP4 antibody.

		Anti-AQP4 antibody - positive patients ($n=12$)	Anti-AQP4 antibody - negative patients ($n=60$)
Age at time of SEP study (years)[a]		49.5 ± 13.4*	39.4 ± 12.3
Time elapsed from disease onset to SEP study (years)[a]		6.8 ± 6.8	8.0 ± 10.3
Past and present history of sensory impairment at time of SEP study		11/12 (91.7%)	54/60 (90.0%)
Superficial sensation in UL[a]		8.3 ± 3.0	7.9 ± 3.5
Superficial sensation in LL[a]		6.6 ± 3.1	7.7 ± 3.3
Vibration sensation in UL[a]		−0.2 ± 0.4	−0.5 ± 1.0
Vibration sensation in LL[a]		−1.9 ± 1.6	−1.4 ± 1.4
Position sensation in UL[a]		−0.4 ± 1.2	−0.3 ± 0.9
Position sensation in LL[a]		−1.4 ± 1.8	−0.6 ± 1.3
Proportion of remission phase		5/12 (41.7%)*	41/60 (68.3%)
IFN beta therapy at time of SEP study		0/12 (0%)	10/60 (16.7%)
Corticosteroid at time of SEP study		11/12 (91.7%)	39/60 (65.0%)
Plasma exchange at time of SEP study		1/12 (8.3%)	2/60 (3.3%)
SEP (CSCT) UL	Unevoked	0/12 (0%)	10/60 (16.7%)
	Delayed	2/12 (16.7%)	8/60 (13.3%)
	Normal	10/12 (83.3%)	42/60 (70.0%)
SEP (CSCT) LL	Unevoked	1/12 (8.3%)	11/60 (18.3%)
	Delayed	2/12 (16.7%)	16/60 (26.7%)
	Normal	9/12 (75.0%)	33/60 (55.0%)

AQP4 = aquaporin-4; CSCT = central sensory conduction time; LL = lower limb; UL = upper limb. *$p<0.05$ in comparison between patients with and without anti-AQP4 antibody.
[a] Mean ± SD.

Table 4
Motor function and MEP findings in MS patients with and without anti-AQP4 antibody.

		Anti-AQP4 antibody - positive patients ($n=13$)	Anti-AQP4 antibody - negative patients ($n=71$)
Age at time of MEP study (years)[a]		49.5 ± 13.7*	38.9 ± 12.3
Time elapsed from disease onset to MEP study (years)[a]		5.3 ± 6.6	7.3 ± 8.4
Past and present history of muscle weakness		12/13 (92.3%)	63/71 (88.7%)
Muscle weakness in UL[a]		−0.8 ± 1.4	−0.7 ± 1.1
Muscle weakness in LL[a]		−1.5 ± 1.2	−1.5 ± 1.3
Proportion of remission phase		6/13 (46.2%)	47/71 (66.2%)
IFN beta therapy at time of MEP study		2/13 (15.4%)	16/71 (22.5%)
Steroid therapy at time of MEP study		11/13 (84.6%)	46/71 (64.8%)
Plasma exchange at time of MEP study		1/13 (7.7%)	2/71 (2.8%)
MEPs (CMCT) UL	Unevoked	5/13 (38.5%)	28/71 (39.4%)
	Delayed	1/13 (7.7%)	16/71 (22.5%)
	Normal	7/13 (53.8%)	27/71 (38.0%)
MEPs (CMCT) LL	Unevoked	4/13 (30.8%)	15/71 (21.1%)
	Delayed	3/13 (23.1%)	25/71 (35.2%)
	Normal	6/13 (46.2%)	31/71 (43.7%)

AQP4 = aquaporin-4; CMCT = central motor conduction time; LL = lower limb; UL = upper limb. *$p<0.05$ in comparison between patients with and without anti-AQP4 antibody.
[a] Mean ± SD.

3.4. MEP findings

The motor functions and MEP findings of the patients are summarized in Table 4. The degree of motor dysfunction was similar between patients with and without anti-AQP4 antibody who were subjected to MEP studies. Frequencies of immunotherapies, such as IFN beta, corticosteroids, and plasma exchange, at the time of study, were not significantly different between anti-AQP4 antibody-positive patients and anti-AQP4 antibody-negative patients. Upper limb MEPs were not evoked in five of the 13 (38.5%) patients with anti-AQP4 antibody and

Table 5
Frequency of MRI lesions in each subgroup classified according to the anti-AQP4 antibody status and evoked potential responses.

		Total	Anti-AQP4 Antibody	
			+	−
VEP (P100)[a]	Unevoked	9/41 (22.2)	4/13 (30.8)	5/28 (17.9)
	Delayed	3/51 (5.9)	1/4 (25.0)	2/47 (4.3)
	Normal	5/104 (4.8)	2/17 (11.8)	3/87 (3.4)
	Subtotal	17/196 (8.7)	7/34 (20.6)	10/162 (6.2)
SEP (CSCT)	Unevoked	14/15 (93.3)	2/2 (100.0)	12/13 (92.3)
	Delayed	16/22 (72.7)	3/3 (100.0)	13/19 (68.4)
	Normal	27/44 (61.4)	4/9 (44.4)	23/35 (65.7)
	Subtotal	57/81 (70.4)	9/14 (64.3)	48/67 (71.6)
MEPs (CMCT)	Unevoked	17/20 (85.0)	3/4 (75.0)	14/16 (87.5)
	Delayed	24/27 (88.9)	3/3 (100.0)	21/24 (87.5)
	Normal	19/39 (48.7)	3/6 (50.0)	16/33 (48.5)
	Subtotal	60/86 (69.8)	9/13 (69.2)	51/73 (69.9)

[a] Number of eyes.

Table 6
Multiple logistic analyses for possible factors contributing to severe visual impairment (finger counting or worse).

Possible factors	Severe visual impairment		OR	95% CI	p value
	+	−			
	(n=20)	(n=81)			
Male	4/20 (20.0%)	22/81 (27.2%)	3.516	(0.557–24.753)	0.182
Age of onset	31.6 ± 13.9[a]	33.6 ± 12.9[a]	0.964	(0.905–1.019)	0.213
Duration from onset to time of study	8.1 ± 6.8[a]	6.7 ± 8.4[a]	0.995	(0.892–1.096)	0.923
Anti-AQP4 antibody (+)	9/20(45.0%)	8 /81 (9.9%)	8.406	(1.547–60.125)	0.02*
Optic nerve MRI lesions	4/20 (20.0%)	7/79 (8.9%)	0.633	(0.074–4.525)	0.659
Unevoked VEP	18/20 (90.0%)	17/81 (20.1%)	35.432	(7.440–284.230)	<0.001*
Corticosteroid treatment at time of study	16/20 (80.0%)	52/81 (64.2%)	2.466	(0.427–18.231)	0.33
Plasma exchange at time of study	1/20 (5.0%)	2/81 (2.5%)	0.837	(0.024–28.101)	0.913
IFN beta therapy at time of study	7/20 (35.0%)	16/81 (19.8%)	0.914	(0.192–3.986)	0.906

*$p<0.05$.
[a] = Mean ± SD; AQP4 = aquaporin-4; CI = confidence interval; OR = odds ratio.

28 of the 71 (39.4%) patients without anti-AQP4 antibody, while one of the 13 (7.7%) patients with anti-AQP4 antibody and 16 of the 71 (22.5%) patients without the antibody showed delays in CMCT. Four of the 13 (30.8%) patients with anti-AQP4 antibody and 15 of the 71 (21.1%) patients without anti-AQP4 antibody showed a lack of evoked lower limb MEPs, while three of the 13 (23.1%) patients with AQP4 antibody and 25 of the 71 (35.2%) patients without the antibody had delayed CMCTs. There was no significant difference in the frequencies of abnormal findings between patients with and without anti-AQP4 antibody.

3.5. MRI findings

The frequencies of MRI lesions in each subgroup according to EP response and anti-AQP4 antibody status are shown in Table 5. Among patients who underwent VEP study, those who showed unevoked VEP responses had a significantly higher frequency of optic nerve lesions on MRI than those who had normal VEP responses (corrected $p=0.0105$). In patients without anti-AQP4 antibody, the frequency of optic nerve lesions on MRI tended to be higher in those with unevoked VEP responses than in those with normal VEP responses (corrected $p=0.0606$). As mentioned above, as a whole, the frequency of optic nerve lesions on MRI was significantly higher in patients with anti-AQP4 antibody than in those without the antibody ($p=0.0137$). The differences in frequencies of optic nerve lesions on MRI among subgroups classified according to VEP responses did not reach statistical significance owing to small sample size. In patients who undertook SEP study, the frequency of spinal cord lesions on MRI tended to be higher in patients with unevoked SEP responses than in those with normal SEP responses (corrected $p=0.0717$). However, no other comparisons between patients with and without anti-AQP4 antibody, or between patients with distinct SEP responses, revealed any significant difference. Among patients who were subjected to MEP study, those who demonstrated unevoked MEP responses and those who had delayed MEP responses had a significantly higher frequency of spinal cord lesions on MRI than those who had normal MEP responses (corrected $p=0.0312$ and 0.0039, respectively). In patients without anti-AQP4 antibody, those with unevoked MEP responses and those with delayed MEP responses showed a significantly higher frequency of spinal cord lesions on MRI than those with normal MEP responses (corrected $p=0.0360$ and 0.0129, respectively). There was no significant difference in the frequency of spinal cord lesions between patients with and without anti-AQP4 antibody.

3.6. Multiple logistic analyses

Among the clinical and laboratory parameters examined, only anti-AQP4 antibody positivity (OR = 8.406, $p=0.02$) and unevoked VEP response (OR = 35.432, $p<0.001$) were significantly related to the occurrence of severe visual impairment (Table 6). Disease duration from onset to time of study (OR = 1.090, $p=0.033$) and unevoked MEP response (OR = 11.013, $p=0.002$) were significantly associated with severe motor weakness in the lower limbs (Table 7). Only unevoked SEP response in the lower limbs was significantly correlated with development of severe sensory impairment in lower limbs (OR = 15.193, $p=0.004$) (other data not shown).

4. Discussion

In the present study, we disclosed that significantly more MS patients with the anti-AQP4 antibody showed a lack of VEP responses than did MS patients without the antibody, whereas delayed latency was more frequently observed in the latter group than in the former. These electrophysiological findings suggest that the nature of the lesions in the optic nerves is necrotic rather than demyelinating in anti-AQP4 antibody-positive patients compared with anti-AQP4 antibody-negative MS patients, which is in good accord with the severe visual impairment in anti-AQP4 antibody-positive patients in the present and previous

Table 7
Multiple logistic analyses for possible factors contributing to severe motor weakness in the lower limbs (moderate or worse).

Possible factors	Severe muscle weakness		OR	95% CI	p value
	+	−			
	(n=36)	(n=46)			
Male	9/36 (25.0%)	18/46 (39.1%)	0.657	(0.177–2.332)	0.518
Age of onset	34.1 ± 14.9[a]	32.2 ± 12.1[a]	1.044	(0.998–1.096)	0.069
Duration from onset to time of study	9.8 ± 9.6[a]	4.9 ± 6.1[a]	1.090	(1.013–1.190)	0.033*
Anti-AQP4 antibody (+)	5/36(13.9%)	7 /46 (15.2%)	0.424	(0.079–2.034)	0.293
Spinal cord MRI lesions	29/36 (80.6%)	29/45 (64.4%)	0.963	(0.285–3.284)	0.951
Unevoked MEP	15/36 (41.7%)	3/46 (6.5%)	11.013	(2.642–63.030)	0.002*
Corticosteroid treatment at time of study	27/36 (75.0%)	29/46 (63.0%)	1.593	(0.447–6.102)	0.478
Plasma exchange at time of study	2/36 (5.6%)	1/46 (2.2%)	1.946	(0.115–57.105)	0.651
IFN beta therapy at study	11/36 (30.1%)	7/46 (15.2%)	3.187	(0.810–13.718)	0.103

*$p<0.05$.
[a] = Mean ± SD; AQP4 = aquaporin-4; CI = confidence interval; OR = odds ratio.

studies [11]. Significant differences in age at onset were observed between anti-AQP4 antibody-positive and -negative patients. However, age at the time of VEP study and elapsed time from disease onset to the study were not different between the two groups. Moreover, multiple logistic analyses revealed that neither age at disease onset nor disease duration was significantly associated with severe visual impairment. Therefore, we do not think that the difference in age at onset significantly contributed to the worsening of VEPs in anti-AQP4 antibody-positive patients. We did not find any difference in the frequencies of abnormal findings in either SEPs or MEPs in our patients according to anti-AQP4 antibody status. This may be explained by the relatively high frequency of severe spinal cord involvement, even in anti-AQP4 antibody-negative MS patients, in Asians [1,12].

Concerning the relationship between EP abnormalities and MRI lesions, in all modalities of EPs, those who showed unevoked EP responses had higher frequencies of MRI lesions in either the optic nerve or spinal cord than those who had normal EP responses, suggesting a good concordance between electrophysiological and neuroimaging abnormalities. Regarding the relationship between anti-AQP4 antibody positivity and MRI lesions, the frequency of the optic nerve lesions on MRI was significantly higher in anti-AQP4 antibody-positive patients than in anti-AQP4 antibody-negative patients. Although the rate of detection of optic nerve lesions was not high by ordinary MRI, the higher frequency of optic nerve MRI lesions in anti-AQP4 antibody-positive patients may well reflect the severe involvement of the optic nerve in this condition. The frequency of all spinal cord lesions, including long and short lesions on MRI, was not different between patients with and without anti-AQP4 antibody; however, the frequency of LESCLs was also significantly higher in patients with anti-AQP4 antibody than in those without the antibody, probably reflecting severe inflammation and associated profuse edema in the spinal cord.

According to the multiple logistic analyses, unevoked VEP, MEP, and SEP responses were significantly correlated with severe visual, motor and sensory impairment, respectively. This indicates that unevoked EP responses reflect severe tissue destruction. Interestingly, among visual, motor and sensory impairments, anti-AQP4 antibody was found to be significantly related to only severe visual impairment by multiple logistic analyses. This suggests that some mechanism is operative to cause grave tissue damage in the optic nerve in patients carrying anti-AQP4 antibody.

We previously reported that the extensive white matter lesions occasionally seen in anti-AQP4 antibody-positive patients show a vasogenic edema pattern on diffusion-weighted MRI, with high signals on apparent diffusion coefficient maps and low signals on diffusion-weighted images [12]. We recently reported marked upregulation of proinflammatory cytokines, such as IL-17, IFN-gamma, TNF-alpha, and IL-8, in the cerebrospinal fluid of patients with the anti-AQP4 antibody [22]. Therefore, in patients harboring the anti-AQP4 antibody, vasogenic edema associated with severe inflammation is likely to occur in the CNS lesions.

AQP4 knock-out mice show amelioration of cytotoxic edema [27] but worsening of vasogenic edema [28] suggesting an important role of AQP4 molecules in the resolution of vasogenic edema. As anti-AQP4 antibody induces down-modulation of AQP4 molecules in AQP4-transfected cultured cells [29], loss of AQP4 on astrocyte foot processes in vivo may retard the resolution of vasogenic edema. If anti-AQP4 antibodies are promptly removed by plasma exchange or extracellular fluid volume is reduced by corticosteroids, such edematous lesions may fade away leaving no major deficits in either myelopathy or encephalopathy, probably through recycling of AQP4 molecules and repair of astrocyte foot processes [29]. By contrast, in the case of the optic nerve, the compensatory space for distension is extremely small, being smallest at the orbital end [30], and the blood is supplied only from the anastomosed small vessels between the dural and pial vessel systems [31]. Therefore, once the optic nerve inflammation extends to the anterior portion of the canal, vasogenic edema accompanied by inflammation easily causes optic nerve ischemia, resulting in severe residual visual impairment [32]. The occurrence of such a secondary ischemic optic neuropathy owing to tissue edema is well known in a variety of conditions, such as minor head trauma, craniofacial surgery, hemorrhage, tumors, and intracranial hypertension [30–34]. Therefore, it is easily conceivable that prolonged vasogenic edema accompanied by the optic neuritis may produce obstruction of small vessels at the optic canal, and cause ischemic necrosis of the optic nerve in anti-AQP4 antibody-positive patients. This assumption may well explain the observation that NMO-IgG is significantly associated only with severe visual impairment [11].

The present study disclosed a high frequency of patients with anti-AQP4 antibody lacking VEP responses, suggesting critical roles for the antibody in severe optic nerve impairment. A delay in the initiation of immunological treatment for this condition may lead to a loss of visual function. These observations thus warrant prompt immunological treatment, such as plasma exchange and intravenous immunoglobulin administration, in patients with anti-AQP4 antibody who show optic nerve involvement.

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Heterogeneity and continuum of multiple sclerosis phenotypes in Japanese according to the results of the fourth nationwide survey

Takaaki Ishizu [a,1], Jun-ichi Kira [a,1,*], Manabu Osoegawa [a], Toshiyuki Fukazawa [b], Seiji Kikuchi [c], Kazuo Fujihara [d], Makoto Matsui [e], Tatsuo Kohriyama [f], Gen Sobue [g], Takashi Yamamura [h], Yasuto Itoyama [d], Takahiko Saida [i], Kiyomi Sakata [j]
and The Research Committee of Neuroimmunological Diseases

[a] Department of Neurology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, 3-1-1Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
[b] Department of Neurology, Nishimaruyama Hospital, Sapporo, Japan
[c] Department of Neurology, Sapporo-Minami National Hospital, Sapporo, Japan
[d] Department of Neurology, Tohoku University School of Medicine, Sendai, Japan
[e] Department of Neurology, Kanazawa Medical University, Kanazawa, Japan
[f] Department of Clinical Neuroscience and Therapeutics, Division of Integrated Medical Science, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan
[g] Department of Neurology, Nagoya University Graduate School of Medicine, Nagoya, Japan
[h] Department of Immunology, National Institute of Neuroscience, NCNP, Tokyo, Japan
[i] Department of Neurology, Center for Neurological Diseases, Utano National Hospital, Kyoto, Japan
[j] Department of Hygiene and Preventive Medicine, Iwate Medical University School of Medicine, Morioka, Japan

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ABSTRACT

There are two distinct phenotypes of multiple sclerosis (MS) in Asians, optic-spinal MS (OSMS) and conventional MS (CMS). In 2004, we performed the fourth nationwide epidemiological survey of MS. The epidemiological features were reported elsewhere; here we report the characteristic features of patients with each MS phenotype, classified according to the clinically estimated sites of involvement and MRI findings. Among 1493 MS patients collated, 57.7% were classified as having CMS and 16.5% were classified as having OSMS. Based on MRI findings, MS patients were further subdivided into those with OSMS with or without longitudinally extensive spinal cord lesions (LESCLs) and those with CMS with or without LESCLs. Although disease duration did not differ significantly among the four groups, EDSS scores were significantly higher in patients with LESCLs than in those without LESCLs, irrespective of OSMS or CMS phenotype. Similar trends were found for the frequencies of bilateral visual loss, transverse myelitis, and marked CSF pleocytosis and neutrophilia. Increased IgG index, brain lesions fulfilling the Barkhof criteria and secondary progression were more commonly found in CMS patients than in OSMS patients, while negative brain MRIs were more commonly encountered in OSMS patients than CMS patients, irrespective of the presence of LESCLs. These findings suggest that demographic features not only vary based on CMS or OSMS phenotype, but also with the presence or absence of LESCLs, and that nonetheless, these four phenotypes constitute a continuum.

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). MS is rare in Asians, but when it appears, involvement of the optic nerve and spinal cord is destructive [1]. There are two distinct subtypes of MS in Asians: the optic-spinal form (OSMS), which shows selective involvement of the optic nerve and spinal cord, and the conventional form (CMS), which affects multiple sites of the central nervous system (CNS), including the cerebrum and cerebellum [2]. The two subtypes have distinct clinical and neuroimaging features. OSMS is characterized by a higher age at onset, greater female preponderance and higher Kurtzke's Expanded Disability Status Scale (EDSS) score [3] compared with CMS [1,2]. Longitudinally extensive spinal cord lesions (LESCLs) extending over three or more vertebral segments are more commonly found in patients with OSMS than CMS patients [1]. However, reflecting the pronounced spinal cord damage seen in Asians, one-fourth of CMS patients also have such LESCLs [4,5].

In Japan, nationwide surveys of MS were conducted in 1972, 1982, 1989 and 2004 using essentially identical criteria [6–8]. In the fourth survey, we disclosed a four-fold increase in the estimated number of clinically definite MS patients (9900; crude MS prevalence, 7.7/100,000) in 2003 compared with 1972, and a shift in the peak age at onset from early 30 s in 1989 to early 20 s in 2003 [8], suggesting an

* Corresponding author. Tel.: +81 92 642 5340; fax: +81 92 642 5352.
E-mail address: kira@neuro.med.kyushu-u.ac.jp (J. Kira).
[1] These authors contributed equally to this work.

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Table 1
Clinical characteristics among each multiple sclerosis subgroups.

	OSMS		CMS	
	LESCL (+)	LESCL (−)	LESCL (+)	LESCL (−)
	(n=93)	(n=117)	(n=121)	(n=570)
Sex ratio (male:female)	1:5.2*k	1:4.1	1:5.1*h	1:2.3*b, *k
Age at onset (years)	38.8 ± 12.8*c, *e, *g	33.2 ± 12.0*d, *g	31.1 ± 14.9*c	29.3 ± 11.9*d, *e
Age at examination (years)	49.8 ± 13.9*c, *e, *g	43.9 ± 13.2*d, *g	41.4 ± 15.6*c	39.7 ± 12.8*d, *e
Disease duration (years)	11.1 ± 8.0	10.6 ± 8.7	10.4 ± 8.8	10.4 ± 8.3
EDSS scores	5.4 ± 2.5*a, *e	3.2 ± 2.5*a, *f	4.9 ± 2.9*b, *f	3.2 ± 2.5*b, *e
Symptoms during entire course				
Bilateral visual loss	57/93 (61.3%)*e	51/117 (43.6%)*d	54/121 (44.6%)*b	142/563 (25.2%)*b, *d, *e
Transverse myelitis	58/91 (63.7%)*a, *e	39/113 (34.5%)*a, *d	57/116 (49.1%)*b	91/552 (16.5%)*b, *d, *e
Paraparesis	64/91 (70.3%)*a, *e	51/113 (45.1%)*a	67/116 (57.8%)*b	203/558 (36.4%)*b, *e
Quadriparesis	21/93 (22.6%)	18/112 (16.1%)*l	37/118 (31.4%)*b, *l	89/561 (15.9%)*b
Sensory impairment below a certain level	66/89 (74.2%)*a, *e	53/108 (49.1%)*a, *d	67/110 (60.9%)*b	130/528 (24.6%)*b, *d, *e
Sphincter disturbance	71/93 (76.3%)*a, *e	61/114 (53.5%)*a, *l	86/120 (71.7%)*b, *l	251/563 (44.6%)*b, *e
Severe motor disability at the time of last examination#	30/89 (33.7%)*g, *e	18/110 (16.4%)*g, *f	43/116 (37.1%)*b, *f	70/534 (13.1%)*b, *e
Secondary progression	7/93 (7.5%)	6/117(5.1%)*l, *j	22/121 (18.2%)*l	88/569 (15.5%)*j
Cerebrospinal fluid findings				
Marked pleocytosis (≥ 50 WBC/mm^3) or neutrophilia (≥ 5 neutrophils/mm^3)	16/79 (20.3%)*a, *e	3/96 (3.1%)*a, *l	17/102 (16.7%)*b, *l	21/511 (4.1%)*b, *e
Increased IgG index	12/45 (26.7%)*e	16/51 (31.4%)*d	29/59 (49.2%)	186/298 (62.4%)*d, *e
Brain MRI findings				
≥ 1 Gd-enhanced lesion or ≥ 9 T2 brain lesions	16/87 (18.4%)*c, *e	22/110 (20.0%)*d, *f	72/112 (64.3%)*c, *f	358/548 (65.3%)*d, *e
≥ 9 T2 brain lesions	13/87 (14.9%)*c, *e	21/110 (19.1%)*d, *f	49/112 (43.8%)*c, *f	281/547 (51.4%)*d, *e
≥ 1 Gd-enhanced lesion	5/79 (6.3%)*c, *e	5/99 (5.1%)*d, *f	43/100 (43.0%)*c, *f	210/481 (43.7%)*d, *e
≥ 1 Juxtacortical lesion	5/85 (5.9%)*c, *e	21/110 (19.1%)*d, *f	46/109 (42.2%)*c, *f	209/536 (39.0%)*d, *e
≥ 3 Periventricular lesions	21/86 (24.4%)*c, *e	34/111 (30.6%)*d, *f	69/114 (60.5%)*c, *f	365/546 (66.9%)*d, *e
≥ 1 Infratentorial lesion	11/87 (12.6%)*c, *e	26/107 (24.3%)*d, *f	69/116 (59.5%)*c, *f	372/559 (66.5%)*d, *e
Lesions fulfilling the Barkhof criteria	7/89 (7.9%)*c, *e	10/109 (9.2%)*d, *f	47/120 (39.2%)*c, *f	280/566 (49.5%)*d, *e
No cranial lesion	49/89 (55.1%)*a, *c, *e	39/109 (35.8%)*a, *d, *f	3/120 (2.5%)*c, *f	10/566 (1.8%)*d, *e
Spinal cord MRI findings				
≥ 1 T2 lesion	93/93 (100%)*a, *e	97/117 (82.9%)*a, *d, *f	121/121 (100%)*b, *f	354/570 (62.1%)*b, *d, *e
LESCL	93/93 (100%)*a, *e	0/117 (0%)*a, *f	121/121 (100%)*b, *f	0/570 (0%)*b, *e
Gd-enhanced lesion	59/75 (78.7%)*a, *e	39/99 (39.4%)*a, *d, *f	72/107 (67.3%)*b, *f	110/532 (20.7%)*b, *d, *e

#: Chair-bound or worse. CMS = conventional form of multiple sclerosis; EDSS = expanded disability status scale of Kurtzke; Gd = gadolinium; LESCLs = longitudinally extensive spinal cord lesions extending 3 or more vertebral segments; N.S. = not significant; OSMS = optic-spinal form of multiple sclerosis.
*a: P< 0.01 (OSMS with LESCLs vs. OSMS without LESCLs), *b: P<0.01 (CMS with LESCLs vs. CMS without LESCLs), *c: P<0.01 (OSMS with LESCLs vs. CMS with LESCLs), *d: P< 0.01 (OSMS without LESCLs vs. CMS without LESCLs), *e: P<0.01 (OSMS with LESCLs vs. CMS without LESCLs), *f: P<0.01 (OSMS without LESCLs vs. CMS with LESCLs), *g: 0.01 ≤ P< 0.05 (OSMS with LESCLs vs. OSMS without LESCLs), *h: 0.01 ≤ P<0.05 (CMS with LESCLs vs. CMS without LESCLs), *i: 0.01 ≤ P< 0.05 (OSMS with LESCLs vs. CMS with LESCLs), *j: 0.01 ≤ P< 0.05 (OSMS without LESCLs vs. CMS without LESCLs), *k: 0.01 ≤ P< 0.05 (OSMS with LESCLs vs. CMS without LESCLs), *l: 0.01 ≤ P< 0.05 (OSMS without LESCLs vs. CMS with LESCLs).

increase in susceptibility to this disease among the younger generation. In this study, a successive decrease in optic-spinal involvement in clinically definite MS patients was also revealed, while the absolute numbers of CMS patients and those with MS-like brain lesions fulfilling the Barkhof criteria were found to increase rapidly with advancing year of birth. Also, the frequency of LESCLs was found to be significantly higher in OSMS patients than in CMS patients in this nationwide survey.

We recently reported that there are distinct subtypes of MS according to clinical and MRI findings using our institutional series of MS patients [9,10]. Therefore, in the present study, we aimed to clarify the characteristic features of each MS phenotype classified according to the clinically estimated sites of involvement and MRI findings unique to Asian MS patients, such as the presence or absence of LESCLs, using collated MS cases from the fourth nationwide survey of MS in Japan.

2. Methods

2.1. Survey procedures

The fourth nationwide survey of MS was conducted by the Research Committees of Neuroimmunological Diseases and of Epidemiology of Intractable Diseases, sponsored by the Ministry of Health, Labor and Welfare, Japan. The study was approved by the Kyushu University Ethics Committee. The survey was undertaken in two steps: first, a preliminary survey was undertaken to ascertain the approximate number of MS patients in Japan, and second, a survey was conducted using a questionnaire sheet for each patient. The hospitals included in the study were randomly selected from the directory of all of the registered hospitals throughout Japan. Selection was made according to a stratification based on the number of beds in each hospital; the more beds a hospital had, the higher was its probability of being selected [11]. Sampling rates were approximately 8%, 13%, 24%, 43%, 83% and 100% for the strata of general hospitals with 20 to 99 beds, 100 to 199 beds, 200 to 299 beds, 300 to 399 beds, 400 to 499 beds and 500+ beds, respectively. All university hospitals, including those in which council members of the Japanese Society of Neurology and members of the Committees of Medical Facilities for Children and the Japanese Society of Child Neurology were working, were also surveyed.

The questionnaire for the preliminary survey on MS patients who visited hospitals because of the disease in 1 year from 1 January to 31 December 2003 was mailed to 6708 departments (including 1933 neurology/internal medicine, 1227 orthopedics, 997 psychiatry, 945 pediatrics, 831 ophthalmology, 759 neurosurgery and 16 rehabilitation departments), together with the diagnostic criteria, in January 2004. In Japan, all patients with MS are requested to visit hospitals at

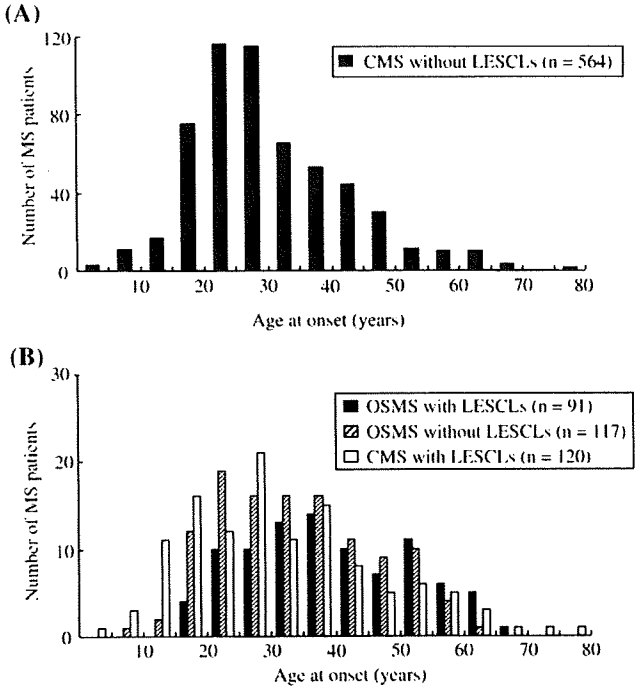

Fig. 1. Distribution of age at onset in patients with CMS without LESCLs (A), CMS without LESCLs, OSMS patients with LESCLs and OSMS patients without LESCLs (B). In (A) and (B), "anticipation" of age at onset is more pronounced in patients without LESCLs, irrespective of CMS or OSMS phenotype. The second peak around the early 50 s is not evident in CMS patients without LESCLs, but is still identifiable in the other three subtypes. CMS = conventional form of multiple sclerosis, LESCLs = longitudinally extensive spinal cord lesions extending three or more vertebral segments on MRI, *n* = number of patients on whom information was obtained, and OSMS = optic-spinal form of multiple sclerosis.

least once every year for registration of intractable diseases with the government in order to have their medical costs, which are not covered by health insurance, subsidized. Following the collection and collation of the first questionnaire, the second questionnaire was forwarded to those institutions reporting patients in the first survey. It requested detailed clinical information on individual patients, including age at onset and examination, sex, birthplace, present address, symptoms based on history and signs based on physical examination, laboratory findings, course, treatment and prognosis. Patients reported by more than one hospital or department were treated as duplicates.

2.2. Diagnostic criteria

The diagnostic criteria used for the present survey were based on those used for the first nationwide survey in 1972 [6], except that the limitation of age at onset was removed, as it was in the third survey [7]. The criteria required multiplicity in time and space and were essentially the same as Schumacher's criteria [12]. Briefly, the criteria used for relapsing remitting multiple sclerosis (MS) in the present survey consisted of three items for clinically definite MS: (1) symptoms and signs owing to multifocal lesions in the CNS (more than two lesions in the CNS); (2) remissions and exacerbations (multiplicity in time); and (3) other diseases, such as tumors, syphilis, cerebrovascular accident, cervical spondylosis, angiomas, subacute myelo-optico-neuropathy, neuro-Behçet, cerebellar degeneration, HTLV-I-associated myelopathy/tropical spastic paraparesis and collagen diseases, could be excluded. Clinically definite MS fulfilled all of the criteria, while a diagnosis of possible MS was made when all three criteria for clinically definite MS could not be fulfilled, but the signs were suggestive. The criteria concerning primary progressive MS (PPMS) were the same as McDonald's criteria [13].

2.3. Classification of clinical phenotype

Clinical classification of MS subtypes was based solely on the clinically estimated sites of the lesions. The second questionnaire asked answerers to report the clinically estimated sites of the lesions according to the symptomatology during the entire clinical course from among the following: optic nerve, cerebrum, cerebellum, brainstem and spinal cord. Moreover, the questionnaire also asked answerers to check for the presence of any of the signs and symptoms listed in the footnote to Table 1, during the entire clinical course. The survey center classified each case into the following clinical subtypes based on the clinically estimated lesion sites reported by each institution: OSMS involving the optic nerve and the spinal cord; optic-brainstem-spinal MS (OBSMS) involving the optic nerve, brainstem and spinal cord; brainstem-spinal MS (BSMS) involving the brainstem and the spinal cord; spinal MS (SMS) involving only the spinal cord, which was identical to recurrent myelitis without any known cause; and conventional MS (CMS), which involved multiple sites of the CNS, including the cerebrum and/or cerebellum. If there was no information about lesion sites, or the symptoms and signs during the entire course were incompatible with the lesion sites, the cases were placed into the unclassified category.

In the preliminary survey, 3749 institutions (55.9%) responded, and reported 4827 MS patients, including 849 patients with possible MS. In the second questionnaire, detailed data were collated for 1919 patients (39.3% of those in the preliminary survey), including 30 duplicate cases. The estimated number of clinically definite MS patients in 2003 was 9900 (95% CI: 9100–10,700) and the estimated crude prevalence was 7.7/100,000 (95% CI: 7.1–8.4) [8]. Based on the clinically estimated sites of lesions, 1493 patients with clinically definite MS and completed questionnaires were classified as having CMS (57.7%), OBSMS (5.8%), BSMS (4.6%), OSMS (16.5%), SMS (10.6%) or unclassified MS (4.9%). In the present study, both CMS and OSMS patients were subjected to further analyses.

2.4. MRI finding-based classification

We recently published a purely MRI findings-based classification in our institutional MS series to minimize the ambiguity of clinical finding-based classification [10]; therefore, we applied such an MRI finding-based classification to the present Japanese nationwide survey series. In the present study, MS patients were classified according to the presence or absence of LESCLs as well as the presence or absence of brain lesions fulfilling the Barkhof criteria (brain lesions fulfilling the Barkhof criteria = Barkhof brain lesions (+)). MS patients were classified into four groups based on MRI findings, Barkhof(+)LESCL(+), Barkhof (+) LESCL(−), Barkhof(−)LESCL(+), and Barkhof(−)LESCL(−), and we compared the demographic features among these groups. To conduct MRI finding-based classification and analyses, longitudinally extensive spinal cord lesions (LESCLs) were defined as those extending over three or more vertebral segments on MRIs taken during the entire clinical course. Fulfillment of the Barkhof criteria [14] was assessed by the central office according to the MRI findings described in the answer sheets. In this analysis, not only patients with OSMS and CMS, but also those with OBSMS, BSMS and SMS, were included.

2.5. Statistical analysis

Statistical analyses of numerical variables were initially performed using the Kruskal–Wallis H test. When statistical significance was found, the Mann–Whitney U test was used to determine the statistical significance of differences between subgroups. Uncorrelated *P* values were corrected by multiplying them by the number of comparisons

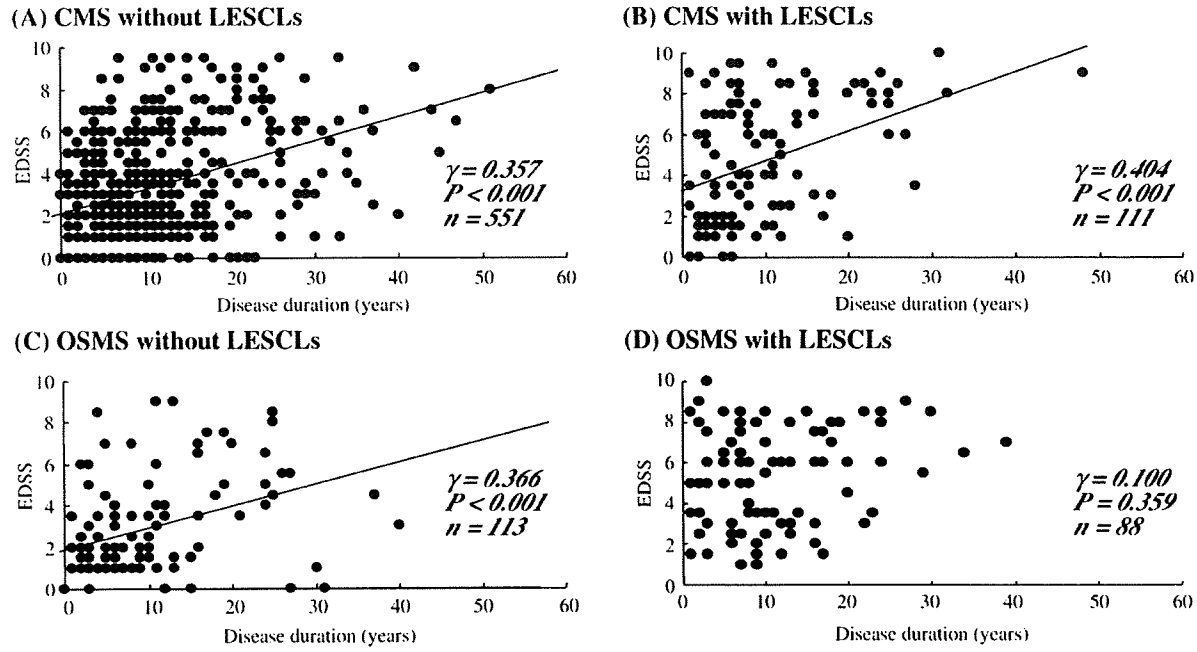

Fig. 2. Relationship between EDSS scores and disease duration in patients with each MS subtype. (A) CMS without LESCLs. (B) CMS with LESCLs. (C) OSMS without LESCLs. (D) OSMS with LESCLs. All patients except OSMS patients with LESCLs show a highly significant correlation between the two parameters. CMS = conventional form of multiple sclerosis, EDSS = Expanded Disability Status Scale of Kurtzke, LESCLs = longitudinally extensive spinal cord lesions extending three or more vertebral segments on MRI, *n* = number of patients on whom information was obtained, and OSMS = optic-spinal form of multiple sclerosis.

(Bonferroni–Dunn's correction) to calculate corrected *P* values. Differences in the ratios between two groups were tested for significance by the χ^2 test or the Fisher's exact test when the criteria for the χ^2 tests were not fulfilled.

3. Results

3.1. Demographic features of each MS subtype

Based on the MRI findings, MS patients were subdivided into those with OSMS with or without LESCLs and those with CMS with or without LESCLs (Table 1). The proportion of females was significantly greater among OSMS or CMS patients with LESCLs than among CMS patients without LESCLs ($P^{corr}<0.05$ in OSMS with LESCLs and $P^{corr}<0.05$ in CMS with LESCLs). Age at onset was significantly higher in OSMS patients with LESCLs than in other patient groups ($P^{corr}<0.05$). The peak age at onset was early 20 s among CMS or OSMS patients without LESCLs, late 20 s among CMS patients with LESCLs, and late 30 s among OSMS patients with LESCLs (Fig. 1A, B). A second peak in the early 50 s was identifiable in all groups except for CMS patients without LESCLs.

Although disease duration did not differ significantly among the four groups, EDSS scores were significantly higher in patients with LESCLs than in those without LESCLs, irrespective of OSMS or CMS phenotype ($P^{corr}<0.01$). Occurrences of bilateral visual loss, transverse myelitis, paraparesis, sensory level and sphincter disturbance were highest in OSMS patients with LESCLs among the four groups ($P^{corr}<0.01$ in all comparisons). CMS patients with LESCLs also showed significantly higher frequencies of these symptoms than CMS patients without LESCLs ($P^{corr}<0.01$ in all). Bilateral visual loss and transverse myelitis were significantly more common in OSMS patients without LESCLs than in CMS patients without LESCLs ($P^{corr}<0.01$ in both). Secondary progression was more common in CMS patients than OSMS patients, regardless of the presence or absence of LESCLs ($P^{corr}<0.05$, OSMS without LESCL vs. CMS with or without LESCL). There was a significant positive correlation between EDSS scores and disease duration in all groups ($P<0.0001$), with the exception of OSMS patients with LESCLs (Fig. 2).

3.2. Laboratory findings in each MS subtype

In the CSF, marked pleocytosis or neutrophilia was more common in patients with LESCLs than in those without LESCLs, irrespective of a diagnosis of OSMS or CMS ($P^{corr}<0.05$ in all). Increased IgG index and brain lesions fulfilling the Barkhof criteria [14] were more commonly found in CMS patients than in OSMS patients, while negative brain MRIs were more commonly encountered in OSMS patients than CMS patients, irrespective of the presence of LESCLs ($P^{corr}<0.01$ in all). Even when MS patients with a disease duration of less than 10 years were excluded, more OSMS patients showed a lack of brain lesions than CMS patients (53.8% of OSMS patients with LESCLs, 34% of OSMS patients without LESCLs, 2.1% of CMS patients with LESCLs, and 2.1% of CMS patients without LESCLs, $P^{corr}<0.01$ in all comparisons), while there were fewer OSMS patients than CMS patients with Barkhof brain lesions (5.1% of OSMS patients with LESCLs, 6.4% of OSMS patients without LESCLs, 51.1% of CMS patients with LESCLs, and 54.4% of CMS patients without LESCLs, $P^{corr}<0.01$ in all comparisons), regardless of the presence or absence of LESCLs. Gadolinium enhancement of the spinal cord lesions was significantly more common in patients with LESCLs than in those without, irrespective of clinical phenotype ($P^{corr}<0.01$ in all).

3.3. Comparison of demographic features among MS patients with contrast-enhanced spinal cord lesions

To focus on inflammatory spinal cord lesions, we compared the demographic features of MS patients with contrast-enhanced spinal cord lesions according to the clinical classification of OSMS or CMS and MRI findings of LESCL positivity. We found essentially the same tendency in this analysis as in the analysis of all the spinal cord lesions, but lost some statistical significance owing to the small sample size (Supplementary Table).

3.4. Comparison of the demographic features of MS patients according to MRI finding-based classification

We finally classified MS patients according to two hallmark MRI findings: brain lesions fulfilling the Barkhof criteria and LESCLs (Table 2). The former is the characteristic feature of Western MS, while the latter is characteristic of Asian MS.

The proportion of females was highest in the Barkhof(+)LESCL(+) group, but no significant difference was found among the four groups. The age at onset was higher in the Barkhof(−)LESCL(+) group than in any other group ($P^{corr}<0.01$ in all comparisons). Although the disease duration was not significantly different among the four groups, the EDSS scores were significantly higher in patients with LESCLs than in those without LESCLs, irrespective of the presence or absence of Barkhof brain lesions ($P^{corr}<0.01$ in all comparisons). Likewise, the frequencies of bilateral visual loss, transverse myelitis, paraparesis, quadriparesis, sensory level and sphincter disturbance were significantly higher in patients with LESCLs than in those without LESCLs, regardless of the presence or absence of Barkhof brain lesions ($P^{corr}<0.05$ in all comparisons). By contrast, the frequency of secondary progression was significantly higher in patients with Barkhof brain lesions than those without Barkhof brain lesions ($P^{corr}<0.05$ in all comparisons). Marked CSF pleocytosis and CSF neutrophilia were more frequent in the Barkhof(−)LESCL(+) group than the Barkhof (+)LESCL(−) and Barkhof(−)LESCL(−) groups ($P^{corr}<0.01$ in all comparisons), while the frequency of increased IgG index was significantly more common in the Barkhof (+)LESCL(−) group than the Barkhof(−)LESCL(+) and Barkhof(−)LESCL(−) groups ($P^{corr}<0.01$ in all comparisons).

4. Discussion

In the present study, using MS cases collated in the fourth nationwide survey in Japan, we disclose that distinct demographic features vary not only with clinical phenotype, such as OSMS and CMS, but also with the characteristic MRI findings, such as LESCLs and Barkhof brain lesions.

The present study had some limitations, primarily because the response rate in the second survey was not high. Concerning the relatively low response rate to this type of nationwide epidemiological survey in Japan, the assumption that the mean number of patients among responding hospitals is equal to that among non-responding hospitals has already been validated [15]. Therefore, we consider that our results would not be distorted seriously by the relatively low response rates. Second, the study was inevitably limited by the fact that the questionnaires were answered by many different clinicians across the country: 88% of the questionnaires were collected from neurologists, 70% of whom had previously participated in a randomized controlled trial of interferon beta-1b [16], which increases the quality of the data, but unfortunately produces a selection bias.

Subtype classification of MS based on symptomatology tends to have some ambiguity and arbitrariness, which may produce equivocal results. To minimize such a limitation, clinical classification was performed in all cases by the central office reviewing collected information. The present survey could not incorporate testing for either neuromyelitis optica (NMO)-IgG or anti aquaporin-4 (AQP4) antibody [17,18], which had not yet been discovered when this survey was initiated. As NMO-IgG, a newly identified marker for NMO [17,18], was also detected in a fraction of Japanese OSMS patients [19], OSMS is claimed to be the same disease as relapsing NMO [20]. However, recent studies from Japan have revealed that about half of OSMS patients with LESCLs are negative for anti-AQP4 antibodies [21,22], and that both NMO-IgG- and anti-AQP4 antibody-positive MS patients frequently have periventricular ovoid lesions in the brain and short spinal cord lesions in addition to LESCLs, suggesting that there is still some overlap between NMO-IgG-positive and -negative MS patients, at least among Japanese [21]. Given that these limitations exist, a nationwide survey collating a large number of Asian MS cases, including MRI findings for the first time, seems to still be relevant, especially cases of CMS, who rarely have NMO-IgG/anti-AQP4 antibody [21].

Table 2
Clinical features among each multiple sclerosis subgroups classified according to the characteristic MRI findings.

	Barkhof MRI lesion (+)		Barkhof MRI lesion (−)	
	LESCL (+)	LESCL (−)	LESCL (+)	LESCL (−)
	($n=64$)	($n=342$)	($n=213$)	($n=491$)
Sex ratio (male:female)	1:4.8	1:2.3	1:3.7	1:3.1
Age at onset (years)	29.5 ± 15.4*c	28.5 ± 11.4*d,*f	37.8 ± 13.8*b,*c, *f	32.0 ± 12.3*b,*d
Age at examination (years)	40.7 ± 15.6*c	39.1 ± 12.4*f	47.3 ± 14.2*b,*c, *f	41.2 ± 12.8*b
Disease duration (years)	11.2 ± 7.7	10.6 ± 8.2*j	9.7 ± 8.2	9.2 ± 7.7*j
EDSS scores	4.7 ± 3.0*a,*e	3.4 ± 2.5*a,*d,*f	4.9 ± 2.6*b,*f	2.7 ± 2.3*b,*d,*c
Symptoms during entire course				
Bilateral visual loss	29/64(45.3%)*a, *k	86/340 (25.3%)*a,*f	87/213 (40.8%)*b,*f	131/481 (27.2%)*b,*k
Transverse myelitis	30/63 (47.6%)*a, *e	47/335 (14.0%)*a, *d,*f	115/207 (55.6%)*b,*f	115/478 (24.1%)*b,*e, *c
Paraparesis	36/61 (59.0%)*e	146/335 (43.6%)*f	133/207 (64.3%)*b,*f	166/481 (34.5%)*b,*d,*c
Quadriparesis	23/62 (37.1%)*a, *e	60/338 (17.8%)*a	53/209 (*25.4%)*b	55/478 (11.5%)*b,*c
Sensory impairment below a certain level	36/59 (61.0%)*a, *e	86/324 (26.5%)*a, *f	127/198 (64.1%)*b,*f	158/455 (34.7%)*b, *b
Sphincter disturbance	44/64 (68.8%)*e	182/340 (53.5%)*d,*f	155/213 (72.8%)*b,*f	192/483 (39.8%)*b, *d, *c
Severe motor disability at the time of last examination#	24/58 (41.4%)*a, *e	48/326 (14.7%)*a,*f	57/203 (28.1%)*b,*f	42/455 (9.2%)*b, *c
Secondary progression	12/64 (18.8%)*k	63/341 (18.5%)*d,*l	21/213 (9.9%)*l	39/491 (7.9)*d,*k
Cerebrospinal fluid findings				
Marked pleocytosis (≥50 WBC/mm^3) or neutrophilia (≥5 neutrophils/mm^3)	5/53 (9.4%)	10/311 (3.2%)*f	35/184 (19.0%)*b,*f	20/420 (4.8%)
Increased IgG index	17/38 (44.7%)	146/220 (66.4%)*d,*f	42/109 (38.5%)*f	101/221 (45.7%)*d

#: Chair-bound or worse, EDSS = expanded disability status scale of Kurtzke; Gd = gadolimium; LESCLs = longitudinally extensive spinal cord lesions extending 3 or more vertebral segments; N.S. = not significant.

*a: $P<0.01$ (LESCLs<+>Barkhof Brain MRI lesions <+>vs. LESCLs<−>Barkhof Brain MRI lesions <+>), *b: $P<0.01$ (LESCLs<+>Barkhof Brain MRI lesions <−> vs. LESCLs<−>Barkhof Brain MRI lesions <+>),*c: $P<0.01$ (LESCLs<+>Barkhof Brain MRI lesions <+> vs. LESCLs<+>Barkhof Brain MRI lesions <−>), *d: $P<0.01$ (LESCLs<−>Barkhof Brain MRI lesions <+> vs. LESCLs<−>Barkhof Brain MRI lesions <−>),*e: $P<0.01$ (LESCLs<+>Barkhof Brain MRI lesions <+> vs. LESCLs<−>Barkhof Brain MRI lesions <−>),*f: $P<0.01$ (LESCLs<−>Barkhof Brain MRI lesions <+>vs. LESCLs<+>Barkhof Brain MRI lesions <−>), *g: $0.01\le P<0.05$ (LESCLs<+>Barkhof Brain MRI lesions <+> vs. LESCLs<−>Barkhof Brain MRI lesions <+>),*h: $0.01\le P<0.05$ ((LESCLs<+>Barkhof Brain MRI lesions <−> vs. LESCLs<−>Barkhof Brain MRI lesions <−>),*i: $0.01\le P<0.05$ (LESCLs<+>Barkhof Brain MRI lesions <+> vs. LESCLs<+>Barkhof Brain MRI lesions <−>), *j: $0.01\le P<0.05$ (LESCLs<−>Barkhof Brain MRI lesions <+> vs. LESCLs<−>Barkhof Brain MRI lesions <−>),*k: $0.01\le P<0.05$ (LESCLs<+>Barkhof Brain MRI lesions <+> vs. LESCLs<−>Barkhof Brain MRI lesions <+>),*l: $0.01\le P<0.05$ (LESCLs<−>Barkhof Brain MRI lesions <+> vs. LESCLs<+>Barkhof Brain MRI lesions <−>).

Although occurrence of LESCLs was more frequent in OSMS patients than in CMS patients, LESCLs were also clearly present in a considerable fraction of Japanese CMS patients. Because not all MRI scans were performed in the relapse phase, the frequency of LESCLs in the present study could have been underestimated. There is also some ambiguity attributed to the fact that MRI films were not assessed centrally in the present study, which was a nationwide survey using questionnaire sheets and not collecting MRI films; however, importantly, the present study disclosed distinctive clinical features associated with MRI findings. LESCLs, regardless of OSMS or CMS phenotype, were related to greater female preponderance, higher EDSS scores and higher frequencies of bilateral visual loss, transverse myelitis and marked CSF pleocytosis and neutrophilia. Even when we compared the demographic features of MS patients with contrast enhancement of spinal cord lesions to focus on the inflammatory types of the lesions, we found practically the same tendency as seen for all spinal cord lesions. On the other hand, increased IgG index and secondary progression were more closely associated with the presence of brain lesions fulfilling the Barkhof criteria [14]. In addition, Barkhof brain lesions were more frequently detected in CMS patients than in OSMS patients, whereas negative brain MRIs were more commonly encountered in OSMS patients than in CMS patients, irrespective of the presence of LESCLs.

Therefore, it is reasonable to classify MS patients according to the clinically estimated sites of lesions, as previously reported, and, additionally, into four subgroups based on the presence or absence of LESCLs. Under such a classification system, OSMS patients with LESCLs represent prototypic Asian-type MS, while CMS patients without LESCLs, most of whom have Barkhof brain lesions, represent classical Western-type MS [1]; these two subgroups are supposed to exist at opposite ends of the MS spectrum. CMS patients with LESCLs shared many features with OSMS patients with LESCLs, while there were differences in age at onset, brain lesion loads, CSF IgG response and secondary progression, assigning this subtype a unique position.

Many features were also found to be common between OSMS patients without LESCLs and CMS patients without LESCLs; however, these subtypes differed in terms of age of onset, brain lesion loads, CSF IgG responses and secondary progression. Moreover, the follow-up periods of patients with intermediate phenotypes were similar to those of prototypic ones, excluding the possibility that shortness of observation periods resulted in apparently intermediate phenotypes. Although some researchers have claimed that OSMS patients without LESCLs are in fact in the early course of CMS [23], on the basis of the results of the present study and our own MS series [9,10], OSMS without LESCLs appears to be a unique subtype in Asians.

It is thus suggested that in between the two extreme ends of the MS spectrum, represented by OSMS with LESCLs and CMS without LESCLs, there exist a considerable number of patients with intermediate phenotypes, such as CMS with LESCLs and OSMS without LESCLs, showing similarities and dissimilarities to these prototypes. Solely MRI finding-based classification also yielded similar results: the Barkhof (+)LESCL(−) group represents Western-type MS and the Barkhof(−)LESCL(+) group represents Asian-type MS, while in between the two exist the Barkhof(+)LESCL(+) and Barkhof(−) LESCL(−) groups. Ikuta et al. [24] investigated a large number of Japanese and American autopsy cases with MS and found OSMS in 47% of the Japanese series, while 13% of the American cases were classified as having OSMS with frequent necrotic lesions pathologically. The results of this study suggest that even in Westerners, cases with OSMS and destructive spinal cord lesions exist with a frequency that should not be ignored.

We recently reported a decrease in peak age at onset in Japanese MS patients over the period of the four nationwide surveys [8]. The present analyses indicate that such "anticipation" of age at onset occurs in patients without LESCLs, irrespective of CMS or OSMS phenotype, but not in those with LESCLs, suggesting that changes in environmental factors associated with modernization may have differentially influenced disease susceptibility in each subtype.

In the present survey series, OSMS patients without LESCLs and CMS patients with or without LESCLs all showed a significant positive correlation between disease duration and EDSS scores, while OSMS patients with LESCLs did not. The absence of a correlation between disease duration and EDSS scores in OSMS patients with LESCLs may in part be explained by the fact that the severity of relapses determines the residual disability in anti-AQP4 antibody-positive MS/NMO patients with rare secondary progression [21], who overlap OSMS patients with LESCLs. Future nationwide surveys incorporating anti-AQP4 antibody assays and central assessment of MRI scans in Japanese will give further insight into the mechanisms underlying the phenotypic differences in MS patients.

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Appendix A

The chairmen of the previous nationwide survey committees were Professors Yoshigoro Kuroiwa (Department of Neurology, Kyushu University; first survey), Akihiro Igata (Third Department of Internal Medicine, Kagoshima University; second survey), and Hiroshi Nishitani (Department of Neurology, National Utano Hospital; third survey). In the fourth survey, in addition to the authors, the following were members of the Research Committee of Neuroimmunological Diseases: Drs. Susumu Chiba (Department of Neurology, School of Medicine, Sapporo Medical University), Yoshitaka Fujii (Department of Surgery II, Nagoya City University Medical School), Susumu Furukawa (Department of Pediatrics, Yamaguchi University School of Medicine), Hideo Hara (Department of Vascular Dementia Research, National Institute for Longevity Sciences, National Center of Geriatrics and Gerontology), Toshirou Hara (Department of Pediatrics, Graduate School of Medical Sciences, Kyushu University), Kinya Hisanaga (Department of Neurology, Miyagi National Hospital), Shu-ichi Ikeda (Department of Neurology, Shinshu University School of Medicine), Shuji Izumo (Division of Molecular Pathology, Center for Chronic Viral Diseases, Graduate School of Medical and Dental Sciences, Kagoshima University), Ryuji Kaji (Department of Neurology, Graduate School of Medicine, Tokushima University), Takashi Kanda (Department of Neurology and Clinical Neuroscience, Yamaguchi University School of Medicine), Shosei Koh (Department of Biomedical Laboratory Sciences, School of Medicine, Shinshu University), Susumu Kusunoki (Department of Neurology, Kinki University School of Medicine), Satoshi Kuwabara (Department of Neurology, Chiba University School of Medicine), Hidenori Matsuo (Division of Clinical Research, Nagasaki Medical Center of Neurology), Hidehiro Mizusawa (Department of Neurology and Neurological Science, Graduate School, Tokyo Medical and Dental University), Tatsufumi Nakamura (Department of Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences, Nagasaki University), Kyoichi Nomura (Department of Neurology, Saitama Medical School), Mieko Ogino (Department of Internal Medicine III (Neurology), Kitasato University School of Medicine), Yoshiro Ohara (Department of Microbiology, Kanazawa Medical University), Mitsuhiro Osame (Department of Neurology and Geriatrics, Kagoshima University School of Medicine), Kohei Ota (Department of Health Science, Faculty of Science, Tokyo University of Science), Jun Shimizu (Department of Neurology, University of Tokyo), Akio Suzumura (Department of Neuroimmunology, Research Institute of Environmental Medicine, Nagoya University), Takeshi Tabira (Department of

Vascular Dementia Research, National Institute for Longevity Sciences, National Center of Geriatrics and Gerontology), Keiko Tanaka (Department of Neurology, Brain Research Institute, Niigata University), Masami Tanaka (Department of Neurology and Clinical Research Center, Nishi-Niigata Chuo National Hospital), Makoto Yoneda (Second Department of Internal Medicine, Faculty of Medical Sciences, University of Fukui), Hiroaki Yoshikawa (Health Service Center, Kanazawa University) and Nobuhiro Yuki (Department of Neurology and Research Institute for Neuroimmunological Diseases, Dokkyo Medical University School of Medicine).

Appendix B. Supplementary data

Supplementary data associated with this article can befound, in the online version, at doi:10.1016/j.jns.2009.01.008.

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Association of the *HLA-DPB1*0501* allele with anti-aquaporin-4 antibody positivity in Japanese patients with idiopathic central nervous system demyelinating disorders

T. Matsushita[1], T. Matsuoka[1], N. Isobe[1], Y. Kawano[1], M. Minohara[1], N. Shi[1], Y. Nishimura[2], H. Ochi[1] & J. Kira[1]

1 Department of Neurology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan
2 Division of Immunogenetics, Department of Neuroscience and Immunology, Kumamoto University Graduate School of Medical Sciences, Kumamoto, Japan

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Correspondence
Jun-ichi Kira, MD, PhD
Department of Neurology
Neurological Institute
Graduate School of Medical Sciences
Kyushu University
3-1-1 Maidashi
Higashi-ku
Fukuoka 812-8582
Japan
Tel: +81 92 642 5340
Fax: +81 92 642 5352
e-mail: kira@neuro.med.kyushu-u.ac.jp

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Abstract

There are two subtypes of multiple sclerosis (MS) in Asians: the opticospinal (OSMS) form that shows a selective involvement of the optic nerve and the spinal cord and the conventional (CMS) form that has disseminated lesions in the central nervous system including the cerebrum, cerebellum and brainstem. Both show distinct human leukocyte antigen (HLA) class II associations. OSMS has similar features to the relapsing form of neuromyelitis optica (NMO) in Western populations. Recently, it was shown that antibodies to aquaporin-4 (AQP4) are specifically detected in NMO patients and in some Japanese patients with OSMS or recurrent optic neuritis or myelitis. To clarify the immunogenetic background of anti-AQP4 antibody production, we studied *HLA-DRB1* and *-DPB1* gene polymorphisms in anti-AQP4 antibody-positive and -negative patients with idiopathic demyelinating diseases, such as MS, recurrent optic neuritis and recurrent myelitis. The phenotypic frequency of the *HLA-DPB1*0501* allele was significantly increased in anti-AQP4 antibody-positive patients (89.5%, odds ratio = 4.8; 95% confidence interval = 1.6–14.3, $n = 38$, $P^{corr} = 0.032$) compared with controls (64.0%, $n = 125$) but not in either anti-AQP4 antibody-negative OSMS (75.0%, $n = 32$) or CMS (69.2%, $n = 52$) patients. There was no significant correlation between any *HLA-DRB1* allele and the existence of anti-AQP4 antibody. These findings suggest that the emergence of anti-AQP4 antibody is reinforced by the presence of the *HLA-DPB1*0501* allele in Japanese.

Introduction

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) that is generally considered to be mediated by myelin-autoreactive T cells. By contrast, neuromyelitis optica (NMO) is characterised by severe and selective involvement of the optic nerves and spinal cord, the latter of which frequently shows longitudinally extensive spinal cord lesions (LESCLs) extending over three or more vertebral segments (1, 2). Recently, a specific immunoglobulin G (IgG) against NMO, designated NMO-IgG, was described (3); its relevant antigen was reported to be aquaporin-4 (AQP4) (4). Because of the high specificity of NMO-IgG and anti-AQP4 antibody, NMO is claimed to be a distinct disease entity with a causal mechanism fundamentally different from MS (2).

In Asians, the selective and severe involvement of the optic nerves and spinal cord is characteristic (5), and there are two distinct subtypes of MS: the opticospinal (OSMS) form, which has similar features to the relapsing–remitting form of NMO in Western populations (1, 6–8) and the

conventional (CMS) form, which is associated with disseminated lesions in the CNS, including the cerebrum, cerebellum and brainstem (6) similar to classical MS in Western populations (5, 6, 9, 10). We have previously shown that both subtypes show distinct human leukocyte antigen (HLA) class II association: CMS is associated with the *HLA-DRB1*1501* allele, as seen in Caucasian patients with MS (6), while OSMS is associated with the *HLA-DPB1*0501* allele (9).

In a selected series of Japanese patients with OSMS, Nakashima et al. (11) reported an NMO-IgG positivity rate of approximately 60%, and OSMS has now been suggested to be NMO (12). However, we reported the existence of anti-AQP4 antibody-positive MS/NMO patients and anti-AQP4 antibody-negative OSMS patients among Japanese and showed differences in the clinical and neuroimaging features between the two groups (13). The former were not responsive to interferon beta (IFNβ)-1b, while the latter were, which may account for the reported favourable response to IFNβ-1b in OSMS patients in a double-blind controlled trial of the drug in Japanese (14). All these findings suggest that patients with anti-AQP4 antibody are distinct from antibody-negative OSMS patients. However, the immunogenetic background for anti-AQP4 antibody production has not been uncovered.

Thus, to shed light on the mechanism responsible for the distinctive features of anti-AQP4 antibody-positive and -negative OSMS patients, the present study aimed to clarify whether the existence of anti-AQP4 antibody is associated with any *HLA-DRB1* and *-DPB1* alleles.

Materials and methods

Patients

Patients enrolled in the present study were chosen from among patients with clinically definite relapsing–remitting MS, as defined by the criteria of Poser et al. (15), who were thoroughly examined at the MS clinic in the Department of Neurology at Kyushu University Hospital during the period 1987–2007. Informed consent was obtained from 104 patients, 27 males and 77 females, all of whose sera and DNA were available. Two patients with recurrent myelitis who had anti-AQP4 antibody were also included for HLA study because recurrent myelitis and optic neuritis are considered to be early manifestations of anti-AQP4 antibody-positive NMO (2). All patients underwent a thorough neurological examination and routine laboratory tests. All were followed up and clinically evaluated at regular intervals in the MS clinic. All patients were residents of Kyushu Island, the southernmost part of mainland Japan. None of the patients were seropositive for human T-cell leukaemia virus type I. All patients had a relapsing–remitting or relapsing–progressive course, and no patients with primary progressive MS were included in the present study. The MS patients were clinically classified into the two subtypes of OSMS and CMS according to the criteria of Kira et al. (6). Briefly, patients who had a relapsing–remitting course and both optic nerve and spinal cord involvement without any clinical evidence of disease in either the cerebrum or the cerebellum were considered to have OSMS. Patients with minor brainstem signs, such as transient double vision and nystagmus, in addition to opticospinal involvement, were included in this subtype. Patients with multiple involvement of the CNS, including the cerebrum and cerebellum, were considered to have CMS. Between March and October 2007, 54 patients with idiopathic CNS demyelinating diseases were referred to our clinic for anti-AQP4 antibody assay. These patients had been found to be positive for the antibody and were referred to us from educational neurology facilities (five university hospitals and six general hospitals) with two or more neurology specialists accredited by the Japanese Society of Neurology. Among these, 14 patients (25.9%) whose detailed clinical and laboratory information was provided were enrolled in the present study after informed consent was obtained. Among the 14 anti-AQP4 antibody-positive patients with idiopathic CNS demyelinating diseases, there were 9 with clinically definite MS meeting the Poser criteria [8 with OSMS and 1 with CMS according to Kira's classification criteria (6)], 2 with recurrent myelitis, 2 with myelitis and 1 with optic neuritis and myelitis.

The diagnosis of the different forms of MS was made before the HLA assay, and thereafter, the diagnosis remained unchanged throughout the study. The anti-AQP4 antibody-positive group consisted of 24 patients with OSMS, 7 with CMS, 4 with idiopathic recurrent myelitis, 2 with myelitis and 1 with optic neuritis and myelitis.

Medical records and magnetic resonance imaging (MRI) films of the patients were analysed retrospectively for the present study. The disability status of the patients was scored according to the expanded disability status scale (EDSS) of Kurtzke (16). Severe optic neuritis was defined as grade 5 or more than 5 on Kurtzke's visual functional scale (16). Acute transverse myelitis was defined according to Fukazawa et al. (17).

Anti-AQP4 antibody assay

Green fluorescent protein (GFP)–AQP4 fusion protein-transfected human embryonic kidney cells (HEK-293) were produced as previously described (13). Briefly, a full-length complementary DNA (cDNA) encoding human AQP4 (AQP4 transcript variant a; GenBank accession number NM_001650) was amplified from a cDNA library generated from commercially obtained human spinal cord mRNAs (Clontech, Mountain View, CA). The polymerase chain

reaction (PCR) product was cloned into the pDONR221 vector (Invitrogen, Carlsbad, CA), and its sequence was confirmed. After sequencing, the AQP4 cDNA was transferred into the pcDNA-DEST53 expression vector (Invitrogen) to produce a GFP–AQP4 fusion protein in HEK-293 cells. HEK-293 cells maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum were seeded at 10,000 cells/well onto eight-well chamber slides (Becton Dickinson, Franklin Lakes, NJ) 24 h before transfection. The cells were transfected with 100 ng/well of the GFP–AQP4 fusion protein expression vector using FuGENE6 Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. At 48 h after transfection, the cells were initially incubated with human serum samples diluted 1:4 with DMEM for 1 h at 37.0°C, without cell fixation, to suppress non-specific reactions between the serum and the HEK-293 cells, washed in phosphate-buffered saline and visualised with an Alexa 594-conjugated goat anti-human IgG antibody (Invitrogen). With the examiners blinded to the origin of the specimens, the anti-AQP4 antibody assay was carried out at least twice for each sample; samples that gave a positive result twice were deemed to be positive. We validated our assay using sera from 98 patients with idiopathic demyelinating diseases, whose NMO-IgG status was predetermined by the Mayo Clinic and found that the specificity of our assay was the same as that of the NMO-IgG assay, the sensitivity of our assay was 1.1-fold higher than that of the NMO-IgG assay and the anti-AQP4 antibody titre showed a significant positive correlation with NMO-IgG titre ($R = 0.7$, $P < 0.001$).

HLA-DRB1 and *-DPB1* typing

The *HLA-DRB1* and *-DPB1* alleles of the subjects were determined by hybridisation between the products of PCR amplification of the *HLA-DRB1* or *HLA-DPB1* genes and sequence-specific oligonucleotide probes, as described previously (18). As controls, 125 unrelated healthy adults were enrolled. All resided in the southern part of Japan from where all our patients with idiopathic demyelinating diseases were recruited. The nomenclature of the *HLA* alleles followed the World Health Organisation Nomenclature Committee for factors regarding the HLA system (19). The odds ratios (OR) were calculated according to the method of Woolf (20).

Statistical analysis

Statistical assessment of the significance of differences was carried out using Fisher's exact probability test to compare categorical variables. The Kruskal–Wallis H test was used for non-normally distributed variables. When statistical significance was found, the Mann–Whitney *U*-test was used to determine the statistical differences between them. Uncorrelated *P* values (P^{uncorr}) were corrected by multiplying them by the number of comparisons (Bonferroni–Dunn's correction) to calculate corrected *P* values (P^{corr}). In all assays, statistical significance was set at $P < 0.05$.

Results

Relationships between anti-AQP4 antibody positivity and clinical findings

We compared demographic features among anti-AQP4 antibody-positive patients, anti-AQP4 antibody-negative OSMS patients and anti-AQP4 antibody-negative CMS patients (Table 1). The proportion of females was significantly greater in the anti-AQP4 antibody-positive ($P^{corr} = 0.0036$) than in the antibody-negative CMS group. The anti-AQP4 antibody-positive patients showed higher ages at onset ($P^{corr} < 0.001$) and higher EDSS scores ($P^{corr} < 0.001$) than anti-AQP4 antibody-negative CMS patients, yet the disease duration was not significantly different between the two. The anti-AQP4 antibody-negative OSMS patients showed intermediate figures for these parameters between the anti-AQP4 antibody-positive patients and the antibody-negative CMS patients.

On MRI, LESCLs were found significantly more frequently in anti-AQP4 antibody-positive patients than in anti-AQP4 antibody-negative CMS patients ($P^{corr} < 0.001$), while brain lesions fulfilling the Barkhof (21) and Paty (22) criteria were significantly more common in anti-AQP4 antibody-negative CMS patients than in anti-AQP4 antibody-positive patients and anti-AQP4 antibody-negative OSMS patients ($P^{corr} < 0.001$, in both cases). Autoantibodies, such as antinuclear antibody and SS-A/B, were found significantly more commonly in anti-AQP4 antibody-positive patients than in anti-AQP4 antibody-negative OSMS and CMS patients ($P^{corr} = 0.030$ and $P^{corr} = 0.024$, respectively). Other autoimmune diseases coexisted significantly more frequently in anti-AQP4 antibody-positive patients than in anti-AQP4 antibody-negative CMS patients ($P^{corr} < 0.001$). Such coexisting autoimmune diseases in anti-AQP4 antibody-positive patients included five with Sjögren syndrome, four with systemic lupus erythematosus (one patient fulfilled the criteria for both systemic lupus erythematosus and Sjögren syndrome), one with rheumatoid arthritis and one with Hashimoto's thyroiditis.

HLA-DRB1 and *-DPB1* alleles

The frequencies of the *DRB1*0401* and *1202* alleles were higher in anti-AQP4 antibody-positive patients than in healthy controls ($P^{uncorr} = 0.0031$ and $P^{uncorr} = 0.0062$, respectively), and those of the *DRB1*0405* and *0901* alleles were lower ($P^{uncorr} = 0.030$ and $P^{uncorr} = 0.019$, respectively), but all of these differences lost statistical significance after a correction was made (Table 2). By contrast, the

Table 1 Demographic features of anti-AQP4 antibody-positive and -negative patients with idiopathic CNS demyelinating diseases

	Anti-AQP4 antibody-positive patients ($n = 38$)	Anti-AQP4 antibody-negative OSMS ($n = 32$)	Anti-AQP4 antibody-negative CMS ($n = 52$)
Number of females/males	35/3 (11.7:1)*	26/6 (4.3:1)	32/20 (1.6:1)*
Age at onset (years)[a]	41.2 ± 16.3*	31.5 ± 12.9	28.7 ± 11.6*
Disease duration (years)[a]	11.7 ± 9.6	15.1 ± 9.4	12.7 ± 9.7
Relapse rate[a]	0.94 ± 0.68	0.71 ± 0.48	0.68 ± 0.53
EDSS score[a]	5.7 ± 2.3*	4.6 ± 3.1	3.6 ± 2.7*
Frequency of symptoms			
Optic neuritis	33/38 (86.8%)*	32/32 (100.0%)**	27/52 (51.9%)*, **
Bilateral optic neuritis	6/34 (17.7%)	9/32 (28.1%)	4/52 (7.7%)
Severe optic neuritis (≥FS 5)	23/35 (65.7%)*	23/32 (71.9%)**	16/52 (30.8%)*, **
Myelitis	36/38 (94.7%)	32/32 (100.0%)*	42/52 (80.8%)*
Acute transverse myelitis	23/35 (65.7%)*	17/32 (53.1%)**	8/52 (15.4%)*, **
Secondary progression	0/38 (0.0%)	2/32 (6.3%)	4/52 (7.7%)
CSF			
Marked pleocytosis (≥50/μL)	3/31 (9.7%)	5/30 (16.7%)	2/48 (4.2%)
Neutrophilia (≥5/μL)	4/30 (13.3%)	4/30 (13.3%)	1/42 (2.4%)
OB	5/26 (19.2%)	6/27 (22.2%)	20/44 (45.5%)
IgG index (≥0.658)[b]	10/23 (43.5%)	10/25 (40.0%)	26/42 (61.9%)
LESCLs during the entire course	31/38 (81.6%)*	16/32 (50.0%)	15/52 (28.9%)*
Barkhof brain lesions	9/32 (28.1%)*	7/32 (21.9%)**	38/52 (73.1%)*, **
Paty brain lesions	18/32 (56.3%)*	13/32 (40.6%)**	48/52 (92.3%)*, **
SS-A/B	11/32 (34.4%)*, **	2/29 (6.9%)*	3/38 (7.9%)**
ANA and/or SS-A/B	19/32 (59.4%)*, **	7/28 (25.0%)*	11/41 (26.8%)**
Other autoimmune diseases	10/36 (27.8%)*	2/32 (6.3%)	0/52 (0.0%)*

ANA, antinuclear antibody; AQP4, aquaporin-4; Barkhof brain lesions, brain lesions fulfilling the Barkhof criteria (21); CMS, conventional form of multiple sclerosis; CNS, central nervous system; CSF, cerebrospinal fluid; EDSS, expanded disability status scale of Kurtzke; FS, Kurtzke's visual functional scale; IgG, immunoglobulin G; LESCLs, longitudinally extensive spinal cord lesions; OB, oligoclonal IgG bands; OSMS, opticospinal form of multiple sclerosis; Paty brain lesions, brain lesions fulfilling the Paty criteria (22).

[a] Mean ± SD.

[b] The upper-normal range of the IgG index was derived from our previous study (6).

*,** $P^{corr} < 0.05$.

frequency of the *HLA-DPB1*0501* allele was significantly higher in patients with anti-AQP4 antibody than in healthy controls, even after correction ($P^{corr} = 0.032$, OR = 4.8; 95% confidence interval = 1.6–14.3) (Table 3). All 10 patients with anti-AQP4 antibody and other autoimmune diseases carried the *HLA-DPB1*0501* allele.

Discussion

The clinical features of the anti-AQP4 antibody-positive patients in the present study, showing severe optic nerve and spinal cord damage, are in good accord with those reported previously in Western populations and in Japanese (2, 13, 23). In the present study, we disclosed that the frequency of the *HLA-DPB1*0501* allele was significantly increased in Japanese anti-AQP4 antibody-positive patients with idiopathic CNS demyelinating diseases but not in anti-AQP4 antibody-negative OSMS patients. In this study, anti-AQP4 antibody-positive patients were enrolled from several sources, which could introduce some selection bias. However, such an enrolment was carried out from accredited neurology facilities in Japan before HLA determination, and we did not exclude any patients after the HLA study. Therefore, we do not consider that this selection bias seriously distorts the present results.

Variable proportions (30–50%) of either NMO or OSMS patients are seronegative for NMO-IgG/anti-AQP4 antibody (3, 10, 13, 23). Anti-AQP4 antibody-positive and -negative OSMS patients differ in clinical and neuroimaging features. The spinal cord lesions in the former preferentially involve the central grey matter of the thoracic spinal cord, while those in the latter affect the whole length of the spinal cord with a holospinal cord involvement pattern in axial plane images (with both grey and white matter regions affected) (13). Brain lesions are more common in anti-AQP4 antibody-positive patients than in antibody-negative patients (13). IFNβ is effective in the anti-AQP4 antibody-negative patient group but not in the antibody-positive patient group (13). Considering these unique features of the anti-AQP4 antibody-positive patients, it is possible that anti-AQP4 antibody, produced primarily or even secondarily following severe tissue destruction, exerts some *in vivo* effects. However, it remains to be elucidated whether anti-AQP4 antibody is a primary cause or secondary modifying factor in this condition.

Table 2 Phenotypic frequencies of HLA-DRB1 alleles in anti-AQP4 antibody-positive and -negative patients with idiopathic CNS demyelinating diseases[a]

DRB1	Controls ($n = 125$)	Anti-AQP4 antibody-positive patients ($n = 38$)	Anti-AQP4 antibody-negative OSMS patients ($n = 32$)	Anti-AQP4 antibody-negative CMS patients ($n = 52$)
0101	18 (14.4)	4 (10.5)	2 (6.25)	4 (7.69)
0301	2 (1.60)	1 (2.63)	1 (3.13)	0 (0.00)
0401	1 (0.80)	5 (13.2)*	0 (0.00)	2 (3.85)
0403	5 (4.00)	1 (2.63)	0 (0.00)	5 (9.62)
0404	0 (0.00)	0 (0.00)	0 (0.00)	3 (5.77)*
0405	35 (28.0)	4 (10.5)*	9 (28.1)	18 (34.6)
0406	9 (7.20)	2 (5.26)	0 (0.00)	6 (11.5)
0407	0 (0.00)	0 (0.00)	0 (0.00)	1 (1.92)
0410	2 (1.60)	0 (0.00)	5 (15.6)*	3 (5.77)
0701	1 (0.80)	0 (0.00)	0 (0.00)	0 (0.00)
0802	9 (7.20)	6 (15.8)	3 (9.38)	5 (9.62)
0803	22 (17.6)	7 (18.4)	2 (6.25)	8 (15.4)
0901	37 (29.6)	3 (7.89)*	3 (9.38)*	5 (9.62)*
1001	0 (0.00)	0 (0.00)	1 (3.13)	0 (0.00)
1101	2 (1.60)	1 (2.63)	2 (6.25)	0 (0.00)
1201	8 (6.40)	3 (7.89)	4 (12.5)	0 (0.00)
1202	3 (2.40)	5 (13.2)*	2 (6.25)	1 (1.92)
1302	17 (13.6)	5 (13.2)	5 (15.6)	3 (5.77)
1401	5 (4.00)	3 (7.89)	2 (6.25)	1 (1.92)
1403	3 (2.40)	1 (2.63)	2 (6.25)	0 (0.00)
1405	2 (1.60)	3 (7.89)	1 (3.13)	3 (5.77)
1406	3 (2.40)	1 (2.63)	1 (3.13)	1 (1.92)
1501	17 (13.6)	9 (23.7)	7 (21.9)	12 (23.1)
1502	32 (25.6)	6 (15.8)	5 (15.6)	9 (17.3)
1602	1 (0.80)	2 (5.26)	1 (3.13)	1 (1.92)

AQP4, aquaporin-4; CMS, conventional form of multiple sclerosis; CNS, central nervous system; OSMS, opticospinal form of multiple sclerosis.
[a] Percentage in parentheses.
* $P^{uncorr} < 0.05$ when compared with the control group.

Systemic autoantibodies are much more frequently found in anti-AQP4 antibody-positive patients than in anti-AQP4 antibody-negative patients [(1, 13) and the present study], suggesting that background-heightened humoral immunity is one of the critical predisposing factors for anti-AQP4 antibody production. Moreover, in Japanese patients, the

Table 3 Phenotypic frequencies of HLA-DPB1 alleles in anti-AQP4 antibody-positive and -negative patients with idiopathic CNS demyelinating diseases[a]

DPB1	Controls ($n = 125$)	Anti-AQP4 antibody-positive patients ($n = 38$)	Anti-AQP4 antibody-negative OSMS patients ($n = 32$)	Anti-AQP4 antibody-negative CMS patients ($n = 52$)
0201	31 (24.8)	11 (28.9)	15 (46.9)*	22 (42.3)*
0202	5 (4.00)	3 (7.89)	2 (6.25)	2 (3.85)
0301	5 (4.00)	2 (5.26)	4 (12.5)	5 (9.62)
0401	17 (13.6)	6 (15.8)	3 (9.38)	1 (1.92)*
0402	23 (18.4)	6 (15.8)	4 (12.5)	12 (23.1)
0501	80 (64.0)	34 (89.5)**	24 (75.0)	36 (69.2)
0601	3 (2.40)	0 (0.00)	1 (3.13)	1 (1.92)
0901	34 (27.2)	4 (10.5)*	4 (12.5)	8 (15.4)
1301	7 (5.60)	0 (0.00)	0 (0.00)	2 (3.85)
1401	6 (4.80)	0 (0.00)	0 (0.00)	0 (0.00)
1601	2 (1.60)	0 (0.00)	0 (0.00)	0 (0.00)
1701	1 (0.800)	0 (0.00)	0 (0.00)	0 (0.00)
2501	0 (0.00)	1 (2.63)	0 (0.00)	1 (1.92)
4101	0 (0.00)	1 (2.63)	0 (0.00)	0 (0.00)

AQP4, aquaporin-4; CMS, conventional form of multiple sclerosis; CNS, central nervous system; OSMS, opticospinal form of multiple sclerosis.
[a] Percentage in parentheses.
* $P^{uncorr} < 0.05$ when compared with the control group.
** $P^{corr} < 0.05$ when compared with the control group.

*HLA-DPB1*0501* gene allele was significantly associated with the presence of anti-AQP4 antibody. It is thus plausible that anti-AQP4 antibody production is in part attributable to a certain genetic background in addition to exaggerated humoral immunity. This allele is known to be associated with Asian-type MS and is the most common *DPB1* allele in Japanese (9, 24, 25), which may explain the frequent occurrence of anti-AQP4 antibody in Japanese OSMS patients. It is also possible that genetic susceptibility to production of the anti-AQP4 antibody may vary from race to race. In addition, because of rarity of MS and related demyelinating disorders in Asians including Japanese, the number of subjects in the present study was not large. Therefore, the present study is considered to be underpowered for detecting any association with other alleles. Some of the alleles showing only uncorrected *P* values with significance in this present study need to be reinvestigated in a future study with a larger number of patients.

In summary, there are anti-AQP4 antibody-positive OSMS patients in whom the frequency of *HLA-DPB1*0501* is increased, suggesting the possibility that development to AQP4 autoimmunity is in part genetically controlled.

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Angiotensin-converting enzyme (ACE) and ACE2 levels in the cerebrospinal fluid of patients with multiple sclerosis

M Kawajiri[1], M Mogi[2], N Higaki[3], T Matsuoka[4], Y Ohyagi[4], K Tsukuda[2], K Kohara[1], M Horiuchi[2], T Miki[1] and JI Kira[4]

Background We reported a reduction in the levels of angiotensin II in cerebrospinal fluid (CSF) from patients with multiple sclerosis (MS).
Objective and methods To clarify the mechanism underlying this reduction, we assayed angiotensin-converting enzyme (ACE) and ACE2 concentrations along with angiotensin II concentrations in CSF samples from 20 patients with MS and 17 controls with non-neurological diseases.
Results ACE levels were significantly elevated in patients with MS compared with controls (48.42 ± 4.84 vs 44.71 ± 3.9 pg/mL), whereas ACE2 levels were significantly reduced (2.56 ± 0.26 vs 2.78 ± 0.24 pg/mL), acting toward a normalization of angiotensin II levels.
Conclusion These results further indicate an alteration of the intrathecal renin-angiotensin system in patients with MS. *Multiple Sclerosis* 2009; **15**: 262–265. http://msj.sagepub.com

Key words: angiotensin-converting enzyme; cerebrospinal fluid; multiple sclerosis; renin-angiotensin system

Introduction

The renin-angiotensin system (RAS) plays an important role in regulating blood volume and systemic vascular resistance. Our previous work has reported that RAS is involved in neural differentiation via signaling downstream of the receptor for the most potent vasoactive substance, angiotensin II [1,2]. Moreover, we recently discovered reduced levels of angiotensin II in cerebrospinal fluid (CSF) from patients with multiple sclerosis (MS), suggesting that the RAS may also be related to the abnormal neural damage and repair processes in MS [3]. However, the mechanism underlying the reduction in angiotensin II levels in the CSF of patients with MS is still unclear. Angiotensin II production was considered to be determined only by the level of its precursor, angiotensinogen, and the catalytic enzymes, renin, which produces angiotensin I from angiotensinogen, and angiotensin-converting enzyme (ACE), which produces angiotensin II from angiotensin I [4]. However, a homologue of ACE, ACE2, was recently discovered. ACE2 cleaves angiotensin II to produce angiotensin-(1–7), which is also biologically active and counterbalances the actions of angiotensin II (Figure 1) [4]. Thus, ACE2 is regarded as another important regulator in the RAS. Although one earlier report described elevation of ACE levels in the CSF of patients with MS [5], nothing is known about the levels of ACE2 in the CSF. We, therefore, aimed to uncover the mechanism underlying the reduction in angiotensin II levels in MS CSF by assaying both CSF ACE and ACE2 levels along with angiotensin II concentration.

[1]Department of Geriatric Medicine, Graduate School of Medicine, Ehime University, Ehime, Japan
[2]Department of Molecular Cardiovascular Biology and Pharmacology, Graduate School of Medicine, Ehime University, Ehime, Japan
[3]Department of Anesthesiology and Resuscitology, Graduate School of Medicine, Ehime University, Ehime, Japan
[4]Department of Neurology, Neurological Institute, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan
Correspondence to: Masakazu Kawajiri, MD, PhD, Department of Geriatric Medicine, Graduate School of Medicine, Ehime University, Shitsukawa, Tohon, Ehime 791-0295, Japan. Email: mz1965kawajj@khc.biglobe.ne.jp
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