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First diagnostic criteria for atopic myelitis with special reference to discrimination from myelitis-onset multiple sclerosis

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ABSTRACT

Objective: To establish the first evidence-based diagnostic criteria for atopic myelitis (AM) enabling it to be discriminated from myelitis-onset multiple sclerosis (MS), which is a difficult differential diagnosis.
Methods: Sixty-nine consecutive AM patients examined from 1996 to 2010 at Kyushu University hospital, who fulfilled the empirical definition of AM (2003), and 51 myelitis-onset MS patients in whom allergen-specific IgE was measured, were enrolled. The first available brain MRI findings were compared between the two. Then, we compared the clinical and laboratory features between the 16 AM cases who did not meet the Barkhof brain MRI criteria for MS after more than 5 years follow-up and 51 myelitis-onset MS cases. Based on the discriminative findings, we established diagnostic criteria for AM and calculated the sensitivity and specificity.
Results: AM patients had a significantly lower frequency of Barkhof brain lesions on baseline MRI than myelitis-onset MS patients. AM patients had a significantly higher frequency of present and/or past history of atopic disease and hyperIgEemia, and higher cerebrospinal fluid levels of interleukin 9 and CCL11/eotaxin, but a lower frequency of oligoclonal IgG bands than myelitis-onset MS patients. Our proposed diagnostic criteria for AM demonstrated 93.3% sensitivity and 93.3% specificity for AM against myelitis-onset MS, with 82.4% positive predictive value and 97.7% negative predictive value.
Conclusion: Our first evidence-based criteria for AM show high sensitivity and specificity, and would be useful clinically.

1. Introduction

Atopic myelitis (AM) is related to atopic diathesis, mainly atopic disorders such as atopic dermatitis, atopic rhinitis, and bronchial asthma [1]. Since we reported the first cases in 1996 [1], similar clinical and even pathologically confirmed cases [2,3] have been reported from other facilities, mainly in Japan with some from Korea and European countries, and its demographic features have gradually been clarified. Repeated nationwide surveys of AM in Japan have revealed that patients with AM most commonly show cervical cord involvement, mainly in the posterior column, preferentially demonstrating sensory impairment in the four limbs, while motor weakness and muscle atrophy were more frequently seen in those with bronchial asthma than in those with other atopic disorders [4,5]. Such features were similar to those reported in 14 AM patients from Korea [6], although a few differences were noted, such as lower prevalence of a history of atopic diseases, thoracic cord preference, and higher frequencies of gadolinium-enhanced lesions compared with nationwide surveys in Japan. In addition, the nationwide surveys investigating AM and atopy-related peripheral neuritis, such as Churg–Strauss syndrome, have revealed that the clinical or laboratory data from approximately a quarter of AM patients indicated the simultaneous involvement of the peripheral nerves, which thus suggests an overlap with Churg–Strauss syndrome [5]. Moreover, we recently reported the distinct immunological features of AM by cytokine assays of cerebrospinal fluid (CSF): CCL11/eotaxin and interleukin 9 (IL9) were specifically increased in AM patients, but not in patients with other causes of myelitis, including multiple sclerosis (MS), Sjögren syndrome, and HTLV-1-associated myelopathy [7]. Moreover, the increase in IL9 and CCL11/eotaxin showed a significant correlation with disease severity [7]. Collectively, these findings suggest that AM has a mechanism fundamentally distinct from that of MS.

We used the empirical definitions of AM in the first and second nationwide surveys in Japan [4,5]; the first survey defined AM as myelitis of unknown cause with either (1) hyperIgEemia plus mite

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antigen-specific immunoglobulin E (IgE) positivity or (2) coexistent atopic diseases [4], while the second survey also permitted IgE specific for other common environmental allergens besides mite antigens [5]. On the one hand, increasing numbers of AM cases have been reported outside Japan [2,3,6,8], while on the other hand, there is a need for early introduction of disease-modifying drugs for MS or even clinically isolated syndrome. Therefore, demand for more evidence-based criteria for AM is growing, to ensure the correct differentiation of AM from myelitis-onset MS as early as possible. In the present study, we first compared the clinical and laboratory features between patients with AM and those with myelitis-onset MS, which is usually the most difficult differential diagnosis for AM, especially in Asians. Second, we established the first diagnostic criteria for AM based on comparisons of the data, to achieve a reasonably high sensitivity and specificity for diagnosis of AM vs. myelitis-onset MS.

2. Subjects and methods

2.1. Subjects

The medical records of all patients who had been referred to the Department of Neurology, Kyushu University Hospital from January 1996 to September 2010 were reviewed. Consecutive patients with AM and MS who met the following criteria were enrolled. For AM patients, the former (2003) empirically defined AM inclusion criteria were used, which defined AM as myelitis of unknown cause, after exclusion of other diseases, with either (1) hyperIgEemia plus allergen-specific IgE positivity for any allergen, or (2) coexistent or past atopic diseases [5]. All MS patients met the 2005 McDonald criteria [9] and those presenting with myelitis at onset (myelitis-onset MS) who had been examined for allergen-specific IgE were exclusively used in the present study. All enrolled AM and MS patients were confirmed to be negative for anti-aquaporin 4 (AQP4) antibodies.

For both disease groups, AM and MS, the existence of myelitis was confirmed by spinal cord magnetic resonance imaging (MRI), motor-evoked potentials (MEPs), somatosensory-evoked potentials (SEPs), or the findings of neurological examinations in the absence of explainable brain MRI lesions, such as exaggerated deep tendon reflexes, motor weakness of the four limbs without involvement of the cranial region, sensory levels explainable for the spinal cord involvement, and Lhermitte's sign. Measurement of allergen-specific IgE and MRI data of the brain and spinal cord to judge dissemination in the space defined in the revised McDonald criteria [9] were mandatory for individuals in both enrolled groups. For all enrolled cases, the following diseases were considered exclusion criteria: collagen-vasculitis, HTLV-1-associated myelopathy, sarcoidosis, neuromyelitis optica, neurosyphilis, parasitic myelitis, cervical spondylotic myelopathy, spinal cord tumor, and spinal vascular malformation. For further discrimination of clinical and laboratory findings between AM and myelitis-onset MS, only AM patients who were followed up and evaluated by brain MRI more than 5 years from their disease onset, and who did not fulfill the Barkhof MRI criteria for MS [10], were used for comparison with myelitis-onset MS. AM and MS patients for whom there were available data for two or all of the three below-mentioned revised positive supporting criteria (1–3) and the negative supporting criterion (4) were used for sensitivity and specificity evaluation. Written informed consent for using clinical information was obtained from all the participants.

2.2. Clinical and immunological tests

Clinical data were collected from the hospital discharge records or the medical records of the outpatient clinic, which included age of onset, disease onset, and disease course. The severity of the clinical manifestation was evaluated at disease onset and at the latest examination using the Expanded Disability Status Scale (EDSS) of Kurtzke [11]. For measurement of allergen-specific IgE, the following allergens were included: *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, cedar pollen, *Candida*, egg white, milk, wheat, rice, soybean, mold, *Anisakis*, animal skins, house dust, and others according to the patients' atopic diseases. All of the enrolled patients were measured for at least nine common environmental allergens including mite antigens. Bronchial asthma, atopic dermatitis, allergic rhinitis, food allergy, and allergic conjunctivitis were regarded as atopic diseases in the present study. The serum level of IgE and the blood eosinophil count were examined, and 240 U/ml and 500/ml were used as the upper normal limits, respectively. The upper normal limits of IL9 and CCL11/eotaxin in the CSF, as measured by a fluorescent bead-based immunoassay, were 14.0 pg/ml and 2.2 pg/ml, respectively, based on a preliminary study of patients with non-inflammatory neurological disease [7]. Measurement of serum anti-AQP4 antibodies was conducted as previously described [12,13]. Any possibility of other diseases was excluded by comprehensive examination of serum antibodies for parasites, serum angiotensin-converting enzyme and lysozyme, serum anti-nuclear antigen antibodies, serum anti-SS-A/B antibodies, anti-HTLV-1 antibodies in serum and CSF, serologic tests for syphilis, *Treponema pallidum* hemagglutination, and by evaluation of chest X-rays, and brain and spinal cord MRI.

2.3. Electrophysiological tests

MEP, SEP, and visual-evoked potentials (VEP) were recorded as described previously [14].

2.4. Magnetic resonance imaging

Fulfillment of the Barkhof criteria [10], the criteria for dissemination in space, was judged for all the participants according to the distribution and number of T2 lesions. All MRI scans were performed as described previously [12,15]. For the evaluation of spinal cord lesions, the initial MRI scan was reviewed. For the evaluation of brain lesions, the brain MRI scan that was initially available and one conducted more than 5 years after disease onset were used.

2.5. Statistical analysis

Statistical analyses of the numerical variables among the patients' demographic features were performed using the Kruskal–Wallis H test. Comparison of ratios between the two groups was conducted using the χ^2 test or Fisher's exact probability test. We did not apply a logistic regression model for the selection of parameters to be included in the new AM diagnostic criteria due to the small sample size. Instead, those clinical or laboratory parameters that were statistically significantly distinct between AM and MS patients were manually included in the new AM diagnostic criteria to obtain high sensitivity and specificity. Sensitivity meant the probability of the new diagnostic criteria exclusively detecting AM cases from the mixed pool of both AM and myelitis-onset MS patients for whom there were sufficient data for evaluation. Specificity meant the probability that the new AM diagnostic criteria would exclude enrolled myelitis-onset MS cases. In addition, the positive predictive value was calculated by dividing the number of AM cases who also met the new AM criteria by the number of all the cases, including MS cases, who fulfilled the new criteria. The negative predictive value was calculated by dividing the number of MS cases who were successfully excluded by the new AM criteria by the number of all the cases who did not meet the new AM criteria. All analyses were performed using JMP 8.0 (SAS Institute, Cary, NC). Statistical significance was set at $p<0.05$.

3. Results

3.1. Comparison of the demographic features between AM and myelitis-onset MS patients

During the study period, there were 69 cases who fulfilled the former (2003) empirical definition of AM [5] (Fig. 1). Among MS cases who met the revised McDonald criteria [9], there were 90 cases whose disease started with spinal cord lesions; among them, 52 cases were measured for serum antigen-specific IgE for common environmental allergens. Because of insufficient data being available for enrollment, one case whose anti-AQP4 antibody status was unknown was removed; this left 51 MS cases for further analyses. Among the primary sorted 69 AM patients and 51 myelitis-onset MS patients, the baseline (initial) brain MRI scans in our department were available in 38 (55.1%) AM and 45 (88.2%) myelitis-onset MS patients (taken 1.3 ± 1.6 years and 2.7 ± 3.0 years from the disease onset, respectively, $p = 0.0808$); the frequency of fulfillment of the Barkhof criteria was significantly lower in AM patients than in myelitis-onset MS patients (0/38 (0.0%) vs. 11/45 (24.4%), respectively, $p < 0.0011$).

Among the 69 AM patients, 26 were followed up for more than 5 years. Eighteen of these patients underwent brain MRI after at least 5 years; one of these fulfilled the Barkhof criteria at that time. Among the other 17 AM patients, one was not eligible because of the lack of serum samples for anti-AQP4 antibody measurement, leaving 16 patients. Among these 16 AM patients, spinal cord involvement was confirmed by spinal cord MRI in 10 patients (62.5%), by MEP/SEP in four patients (25.0%), and by clinical evaluation in the other two patients (12.5%), one of whom had an exaggerated tendon reflex in four limbs, motor weakness of limb muscles without involvement of the cranial region, Lhermitte's sign and a sensory level, while the other had exaggerated tendon reflex in four limbs, motor weakness of limb muscles without involvement of the cranial region, and a sensory level.

The demographic features of the enrolled 16 AM and 51 MS patients are shown in Table 1. There was no patient who had undergone a spinal cord biopsy who was followed up for more than 5 years. Although the AM patients comprised both genders almost equally and the MS group comprised a larger percentage of females, there was no significant difference between the two. The age of onset for both disease groups was, on average, the early to middle fourth decade. AM patients were significantly more likely to have a current or past history of atopic disease at the time of disease onset compared with myelitis-onset MS patients. Chronic or step-wise onset of the disease was most common in AM, while acute or subacute onset was predominant in myelitis-onset MS. Patients with a monophasic disease course tended to occur more frequently in the AM group than in the myelitis-onset MS group, while those with a relapsing or fluctuating course were significantly more likely to have myelitis-onset MS. The disease duration and EDSS scores at disease onset or at the most recent examination were similar in the two groups. The serum level of total IgE was significantly higher in AM patients than in MS patients, while the blood eosinophil counts were not different between the two groups. For patients whose CSF was examined, the levels of IL9 and CCL11/eotaxin were significantly higher in AM patients than in MS patients. Oligoclonal IgG bands (OCB) were seen in 30.4% of myelitis-onset MS patients but none of the AM patients. There was no significant difference in the frequency of MEP central abnormalities in upper extremities and VEP abnormalities between the AM group and the myelitis-onset MS group. Spinal cord MRI revealed that posterior column lesions in the cervical spinal cord were detected at a similar frequency in both groups.

3.2. Establishment of the diagnostic criteria for AM

Based on the above-mentioned comparison data of the clinical, immunological, electrophysiological, and MRI parameters between the AM and MS groups, we have generated the first evidence-based diagnostic criteria for AM (Table 2). As absolute criteria, in addition to myelitis of unknown etiology excluding diseases mentioned in the footnote to Table 2, we adopted serum positivity for IgE specific to common environmental allergens, plus negativity for brain MRI lesions fulfilling the Barkhof criteria for MS, because these two items showed a statistically significant difference in frequency between AM patients and myelitis-onset MS patients. Although there was no patient with data from a spinal cord biopsy in the present series, we regarded the existence of perivascular lymphocyte cuffings with various degrees of eosinophil infiltration as the pathological criteria, according to previous pathological reports [16,17]. Our supporting

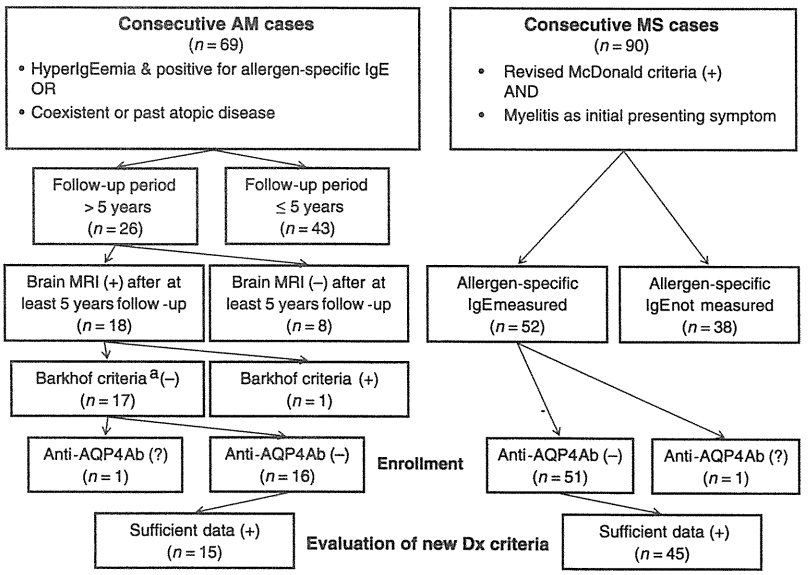

Fig. 1. Enrollment of patients with atopic myelitis (AM) and those with myelitis-onset multiple sclerosis (MS). From consecutive patients with these diseases, 16 AM cases and 51 myelitis-onset MS patients were enrolled for comparison of their demographic features. After establishing the new AM criteria, patients with sufficient data were selected to test the efficacy of the criteria. [a]Barkhof et al. [10]. Ab, antibodies; Dx, diagnostic.

Table 1
Demographic features of enrolled patients with either atopic myelitis or multiple sclerosis.

	AM[a]	Myelitis-onset MS[b]	*p* value
Number of patients	16	51	NA
Gender (male/female)	8/8 (1:1.00)	16/35 (1:2.19)	NS
Age of onset (years old)	35.3 ± 10.3	32.4 ± 11.5	NS
Disease duration (years)	9.1 ± 3.5	10.4 ± 8.1	NS
Present or past history of atopic diseases	14/16 (87.5%)	22/50 (44.0%)	0.0032
Clinical manifestations			
Disease onset			
Acute	2/16 (12.5%)	16/51 (31.4%)	NS
Subacute	2/16 (12.5%)	23/51 (45.1%)	0.0204
Chronic/step-wise	12/16 (75.0%)	12/51 (23.5%)	0.0006
Disease course			
Monophasic	2/15 (13.3%)	0/49 (0.0%)	0.0521
Relapsing/fluctuating	10/15 (66.7%)	44/49 (89.8%)	0.0456
Progressive	3/15 (20.0%)	5/49 (10.2%)	NS
EDSS score at the initial attack	4.1 ± 1.6	3.6 ± 2.1	NS
EDSS score at the final follow-up	3.1 ± 1.4	3.3 ± 2.6	NS
Blood or serum findings			
Blood eosinophil count (/ml)	383.4 ± 355.6	303.8 ± 395.6	NS
Hypereosinophilia[c] (+)	3/16 (18.8%)	9/46 (19.6%)	NS
Serum total IgE (U/ml)	1762 ± 3255	833 ± 4250	<0.0001
HyperIgEemia[d] (+)	12/16 (75.0%)	13/50 (26.0%)	0.0008
Allergen-specific IgE (+)	16/16 (100.0%)	27/51 (52.9%)	0.0003
CSF findings			
IL9 (pg/ml)	15.2 ± 4.7	9.8 ± 3.2	0.0357
Increased IL9[e] (+)	5/8 (62.5%)	1/8 (12.5%)	NS
CCL11/eotaxin (pg/ml)	4.1 ± 0.3	2.0 ± 0.5	0.0008
Increased CCL11/eotaxin[f] (+)	8/8 (100.0%)	3/8 (37.5%)	0.0256
OCB (+)	0/15 (0.0%)	14/46 (30.4%)	0.0137
Electrophysiological findings			
MEP central abnormalities in upper extremities	9/15 (60.0%)	19/36 (52.8%)	NS
VEP abnormalities	3/11 (27.3%)	16/39 (41.0%)	NS
MRI findings and others			
Cervical cord lesions in posterior column	4/16 (25.0%)	12/49 (24.5%)	NS

[a]Those who fulfilled the former (2003) empirical inclusion criteria for AM and were followed up for more than 5 years.
[b]MS patients presenting with myelitis as the initial symptom and who fulfilled the revised McDonald criteria [9].
[c]≥500/ml.
[d]≥240 U/ml.
[e]≥14.0 pg/ml.
[f]≥2.2 pg/ml.
AM = atopic myelitis; CSF = cerebrospinal fluid; EDSS = Expanded Disability Status Scale of Kurtzke; IgE = immunoglobulin E; IL = interleukin; MRI = magnetic resonance imaging; MEP = motor-evoked potential; MS = multiple sclerosis; NA = not applicable; NS = not significant; OCB = oligoclonal bands; VEP = visual-evoked potential.

criteria comprise the following three supporting positive findings: 1) present or past history of atopic disease; 2) serum hyperIgEemia; and 3) increased level of IL9 or CCL11/eotaxin in the CSF, and the following one supporting negative finding: no OCB in the CSF.

Definite AM is therefore defined as follows: (1) cases who meet the absolute criteria plus the pathological criteria, or (2) those who meet the absolute criteria plus two or all of the three supporting positive criteria plus the one supporting negative criterion. Probable cases of AM are defined in Table 2.

3.3. Sensitivity and specificity of the new diagnostic criteria for AM

To test the efficacy of the new AM diagnostic criteria, we selected the cases with sufficient data to judge the fulfillment of the new criteria from the same set of patients. As shown in Fig. 1, 15 AM cases and 45 myelitis-onset MS cases were enrolled. When we applied these AM diagnostic criteria to both the AM cases and myelitis-onset MS cases, 14 (93.3%) of the 15 AM cases met the definite criteria while three of the 45 myelitis-onset MS cases (6.7%) fulfilled the criteria (Table 3-1). Therefore, the sensitivity of this AM criteria was 93.3% and the specificity was 93.3%. Moreover, the positive predictive value was 82.4% while the negative predictive value was 97.7% (Table 3-2).

Table 2
New diagnostic criteria for atopic myelitis.

Criteria	
Absolute criteria	All three of the following are essential. 1) Myelitis with unknown etiology[a] 2) Positive for allergen-specific IgE 3) Negative for Barkhof brain MRI lesions[b]
Pathological criteria	Existence on spinal cord biopsy samples of perivascular lymphocyte cuffings with various degrees of eosinophil infiltration, sometimes accompanied by granuloma.
Supporting criteria Positive findings	1) Present and/or past history of atopic disease 2) Serum hyperIgEemia (≥240 U/ml) 3) Increased level of IL9 (≥14.0 pg/ml) or CCL11/eotaxin (≥2.2 pg/ml) in the CSF
Negative findings	4) No OCB in the CSF
Diagnosis	
Definite	1) Absolute criteria + Major pathological criteria OR 2) Absolute criteria + two or all of the Supporting criteria (1–3) + the Supporting criterion (4)
Probable	1) Absolute criteria + one of the Supporting criteria (1–3) + the Supporting criterion (4) OR 2) Absolute criteria + two or all of the Supporting criteria (1–3)

CSF = cerebrospinal fluid; IgE = immunoglobulin E; IL = interleukin; MEP = motor-evoked potential; MRI = magnetic resonance imaging; OCB = oligoclonal IgG bands; SEP = sensory-evoked potential.
[a]The presence of myelitis should be confirmed by neurologically abnormal sign(s) (limb hyperreflexia and/or sensory levels), MEP, and/or SEP abnormalities suggestive of central nervous system lesions, or spinal cord lesions on MRI. The following diseases should be excluded: parasitic myelitis, multiple sclerosis, collagen-vascular diseases, HTLV-1-associated myelopathy, sarcoidosis, neuromyelitis optica, neurosyphilis, cervical spondylotic myelopathy, spinal cord tumor, spinal vascular malformation.
[b]Barkhof et al. [10].

4. Discussion

This study is the first to compare clinical and laboratory findings between patients with AM and those with myelitis-onset MS, who were all seronegative for anti-AQP4 antibodies. The neurological features of the enrolled AM patients were similar to those in previous nationwide surveys [4,5]. In the present study, we found that,

Table 3-1
Application of the new diagnostic criteria for atopic myelitis.

	AM ($n=15$)	Myelitis-onset MS ($n=45$)
Fulfillment of the new AM diagnostic criteria		
(+)	14	3
(−)	1	42

Table 3-2
Utility of the new diagnostic criteria for atopic myelitis.

Sensitivity	93.3%
Specificity	93.3%
Positive predictive value	82.4%
Negative predictive value	97.7%

AM = atopic myelitis; MS = multiple sclerosis.

compared with myelitis-onset MS patients, AM patients were significantly more likely to have a present and/or past history of atopic disease, serum hyperIgEemia, and allergen-specific IgE, and showed significantly higher levels of IL9 and CCL11/eotaxin in the CSF. By contrast, OCB was significantly less frequent in AM patients than in myelitis-onset MS patients. Moreover, before filtering empirically diagnosed AM cases with the Barkhof criteria, the frequency of fulfillment of the Barkhof criteria at baseline (first available) MRI was found to be significantly lower in AM patients than in myelitis-onset MS patients (0.0% vs. 24.4%, $p<0.0011$). Therefore, it was considered reasonable to incorporate these items, reflecting the characteristic features of each condition, into the first evidence-based diagnostic criteria.

Although blood eosinophilia is one of the distinctive features of Churg–Strauss syndrome [5], the frequency of blood eosinophilia was similar between AM patients and myelitis-onset MS patients. Thus, this was not included in the present criteria. In the present study, we did not find a statistically significant difference for VEP between the two study groups. We consider that this is partly because myelitis-onset MS was used as a disease control, which was expected to have a relatively low frequency of optic nerve involvement early in the course of illness. In fact, we previously reported that the frequency of VEP abnormalities in our anti-AQP4 antibody-seronegative MS patients was around 60% when all cases were used, regardless of the onset sites [14], while in the present study only 41% of myelitis-onset MS patients had abnormal VEPs. In addition, Constantinescu et al. [18] reported a case of atopic optic neuritis, while we also previously reported that a significant fraction (21.7%) of AM patients had VEP abnormalities in the second nationwide survey [5]. Thus, the observation that 27.3% of AM patients had abnormal VEP findings might reflect such a clinically overt or subclinical involvement of optic nerve in this condition, thereby partly contributing to the absence of statistical significance in the comparison of abnormal VEP frequency between AM and myelitis-onset MS patients. For these reasons, we decided not to include the absence of VEP abnormality in the supporting negative criteria for AM. The frequency of posterior column lesions in the cervical cord on MRI was similar in both AM and myelitis-onset MS patients. This is probably explained by the fact that the cervical posterior column is also one of the preferential sites of spinal cord involvement in MS [12,19,20]. Therefore, we did not adopt cervical posterior column lesions as a supporting item in the present criteria.

These first criteria for AM achieved a relatively high sensitivity and specificity against myelitis-onset MS. Occasionally, spinal cord attacks in MS demonstrate neurological features indistinguishable from those of AM. Therefore, in the early stages of MS, especially myelitis-onset MS, it is critical to differentiate MS from AM using certain laboratory markers, because the early use of disease-modifying drugs, such as interferon-beta, is increasingly demanded. Interferon-beta or glatiramer acetate may worsen AM via induction of an immune shift toward a T helper (Th) type 2 cell response [21,22], which plays a key role in atopic disorders [7,23–25]. The high sensitivity and specificity of the present criteria may well facilitate the early discrimination of AM and myelitis-onset MS and contribute to better treatment for both diseases.

The present study has some limitations. First, because of the low prevalence of AM, it was difficult to obtain sufficient cases for enrollment. The paucity of AM patients enrolled in the present study might also have partly influenced the achievement of the surprisingly high negative predictive value and the relatively low positive predictive value of the new AM criteria. Second, for the same reason, we could not evaluate the efficacy of the new AM diagnostic criteria in a replicate population. In the future, the new AM criteria should be tested in other AM cohorts in the Japanese and other ethnic groups. Third, we did not apply a logistic regression model for the selection of parameters to be included in the new AM diagnostic criteria due to the small sample size. Multiple logistic analyses are needed in future large scale studies to identify more specific factors to be incorporated into the diagnostic criteria. Finally, the measurement of IL9 and CCL11/eotaxin in the CSF is not commonly undertaken. Thus, in the new AM criteria, diagnosis of definite AM is designed to be feasible without measuring CSF IL9 or CCL11/eotaxin; however, if measured, elevated levels of these cytokines in the CSF are strongly indicative of AM [7].

Because the prevalence of atopic diseases is rapidly increasing worldwide against a background of improved hygiene, more AM patients might emerge. The first diagnostic criteria for AM will encourage early differential diagnosis of AM and myelitis-onset MS.

Conflict of interest

T.M. received a grant and payment for manuscript preparation and development of educational presentations from Bayer Schering Pharma, and also received a payment for development of educational presentations from Mitsubishi Tanabe Pharma. J.K. is an advisory board member for Merck Serono and a consultant for Biogen Idec Japan. He has received payment for lectures from Bayer Schering Pharma, Cosmic Cooperation and Biogen Idec Japan. This work was supported in part by a Health and Labour Sciences Research Grant on Intractable Diseases (H22-Nanchi-Ippan-130 and H23- Nanchi-Ippan-017) from the Ministry of Health, Labour, and Welfare, Japan, and a Scientific Research B Grant (No. 22390178) and a Challenging Exploratory Research Grant (No. 23659459) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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Clinical disability progression and platelet GP IIb/IIIa values in patients with atopic myelitis

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ABSTRACT

We aimed to clarify the disability progression and platelet aggregative function in atopic myelitis (AM). Seventeen AM patients and 35 healthy controls were subjected to clinico-allergological evaluations and glycoprotein IIb/IIIa (GP IIb/IIIa) measurements using a VerifyNow assay system. In AM patients, the disease duration had significant positive correlations with the Kurtzke Expanded Disability Status Scale scores and Sensory Functional Scale scores. The GP IIb/IIIa values were significantly higher in AM patients than in controls as well as in females compared with males. AM is essentially a progressive disease affecting the sensory system, and involves an increased platelet aggregative function.

1. Introduction

We first reported the emergence of myelitis in patients with atopic disorders, and named it atopic myelitis (AM) (Kira et al., 1997, 1998, 1999, 2001). Repeated nationwide surveys of this condition have revealed a widespread occurrence of AM in Japan (Osoegawa et al., 2003a; Isobe et al., 2009). Similar cases have recently been reported in Europe (Zoli et al., 2005; Constantinescu et al., 2006), including a biopsy-proven case showing marked eosinophil infiltration (Gregoire et al., 2006), as well as in East Asia, including a relatively large series from Korea (Yoon et al., 2009). In AM patients, we found that CCL2, a chemokine for eosinophils, and interleukin-9, a T helper 9 cytokine, were both markedly upregulated in the cerebrospinal fluid, and that the levels of these molecules showed strong positive correlations with the disease severity (Tanaka et al., 2008), collectively suggesting that atopy-related inflammation is operative. A histological study of biopsied spinal cord specimens revealed eosinophilic inflammation and simultaneous loss of both axons and myelin (Kikuchi et al., 2001; Osoegawa et al., 2003b). The condition showed a poor response to corticosteroids but responded to plasma exchanges (Murai et al., 2004). However, the disability progression over the clinical course is still ill-defined.

A recent nationwide survey investigating both AM and atopy-related peripheral neuritis (APN), such as Churg–Strauss syndrome (CSS), revealed a considerable overlap between AM and APN (Isobe et al., 2009). In CSS, ischemia of peripheral nerves caused by inflammation is supposed to be the dominant mechanism for neural damage, and even the optic nerve is affected by the ischemic process in this condition (Liou et al., 1994; Giorgi et al., 1997). Atopic disorders have been reported to be associated with cardiovascular diseases (Brunekreef et al., 2000), and platelet activation in allergy is assumed to play a significant role in these situations (Masini et al., 1994). Platelet aggregation is mediated by interactions of fibrinogen with glycoprotein receptors on platelets, such as glycoprotein IIb/IIIa (GP IIb/IIIa) (αIIbβ3 integrin), which is the central receptor for platelet aggregation (Kasperska-Zajac and Rogala, 2007; Pitchford, 2007). Therefore, in the present study, we aimed first to clarify the relationship between the disease duration and disability progression in AM, and second to reveal any platelet aggregative function abnormalities by measuring the GP IIb/IIIa contents.

2. Subjects and methods

2.1. Subjects and clinico-allergological evaluation

AM was defined as myelitis of unknown cause with either (1) hyperIgEemia (>240 U/ml) and antigen-specific IgE positivity or (2) coexistent or past atopic diseases following the diagnostic criteria,

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excluding other diseases (Osoegawa et al., 2003a). Bronchial asthma, atopic dermatitis, allergic rhinitis, food allergy and allergic conjunctivitis were regarded as atopic diseases in the present study. The existence of myelitis was confirmed by spinal cord MRI, motor-evoked potentials, somatosensory-evoked potentials or neurological findings of either exaggerated deep tendon reflexes or sensory levels. Detailed clinical information on individual patients, including symptomatology, disability scores including the Kurtzke Expanded Disability Status Scale (EDSS) score (Kurtzke, 1983), Pyramidal Functional Scale (FS) score (Kurtzke, 1983) and Sensory FS score (Kurtzke, 1983), Progression Index (Sanders et al., 1986; Chapman et al., 2001), and allergological, neuroimaging and electrophysiological data, were retrospectively evaluated. All 17 AM patients who visited the Department of Neurology, Kyushu University Hospital, from 1 March 2010 to 31 May 2011 were enrolled in the present study, with no medications within 1 week prior to measurement. The AM patients comprised 6 males and 11 females, with a mean (±SD) age at examination of 43.4±13.2 years, mean age at onset of 36.3±12.2 years and disease duration of 7.0±5.0 years. In addition, 35 healthy controls with no medication were evaluated in this study. The control subjects comprised 16 males and 19 females, with a mean age at examination of 31.6±4.8 years. The sex ratios did not differ significantly between the two groups, while the age at examination was significantly higher in the AM patients than in the controls ($p<0.01$). All the AM patients and controls were subjected to a questionnaire survey for past and present history of the above-mentioned atopic diseases, and underwent routine laboratory tests including blood cell counts (white blood cells, platelets, eosinophils, neutrophils and lymphocytes), hemoglobin, total IgE and common allergen-specific IgE for *Dermatophagoides pteronyssinus* (*Dp*) and *Dermatophagoides farinae* (*Df*). *Dp* was measured in all 17 AM patients, while *Df* was examined in 16 AM patients. This study was approved by the ethical committee of Kyushu University Hospital. Written informed consent was obtained from all subjects.

2.2. Measurement of GP IIb/IIIa

GP IIb/IIIa was assayed as an index of platelet aggregative function using a VerifyNow GP IIb/IIIa assay system (Accumetrics, San Diego, CA; Van Werkum et al., 2008). This spectrophotometric assay system is comparable to other well-established methods for platelet aggregation and produces rapid results with small amounts of whole blood (Matzdorff et al., 2001; Wheeler et al., 2002; White et al., 2004). Fresh venous blood was drawn from the patients and healthy controls, who had received no medications affecting platelet aggregation at least for 1 week prior to the blood drawing, and immediately subjected to the assay according to the manufacturer's recommendations (Accumetrics; Michelson, 2009). The results were expressed as platelet aggregation units (PAU).

2.3. Statistical analysis

First, we examined whether all of the clinical and laboratory data showed normal distributions. Student's *t*-test and Welch's test were used to evaluate the significance of differences between the laboratory and demographic features between the AM patients and controls. When comparing the frequencies of atopic disorders between the AM patients and controls, Fisher's exact probability test was used. Since the GP IIb/IIIa values showed a normal distribution in the subjects, a two-way ANOVA was used to compare the GP IIb/IIIa values by sex and disease. Pearson's r correlation test was used to measure the degrees of the relationships between the GP IIb/IIIa values and clinical and laboratory parameters. The level of statistical significance was set at $p<0.05$. All analyses were performed using SPSS software (SPSS Inc., Chicago, IL).

3. Results

3.1. Demographic features of the AM patients

The AM patients showed EDSS scores of 3.2±1.8 (mean±SD), Pyramidal FS scores of 2.2±1.3, Sensory FS scores of 1.9±1.5 and Progression Indexes of 1.3±1.7. The disease duration showed significant positive correlations with the EDSS scores ($r=0.61$, $p<0.01$) and Sensory FS scores ($r=0.64$, $p<0.01$), but not the Pyramidal FS scores (Fig. 1A, B). There were no sex differences in any of the clinical parameters (data not shown).

3.2. Comparisons of hematological and allergological findings between the AM patients and healthy controls

Compared with the controls, the AM patients had significantly higher frequencies of bronchial asthma ($p<0.001$), allergic rhinitis ($p<0.05$), food allergy ($p<0.05$) and allergic conjunctivitis ($p<0.05$) (Table 1). The IgE levels and neutrophil counts were significantly higher in the AM patients than in the controls ($p<0.05$ for both). The allergen-specific IgE levels did not differ significantly between the AM patients and controls in the present study, including those against *Dp* and *Df*, which probably reflects the small sample size. There were no other significant differences in the routine hematological tests between the two groups. The hemoglobin levels were

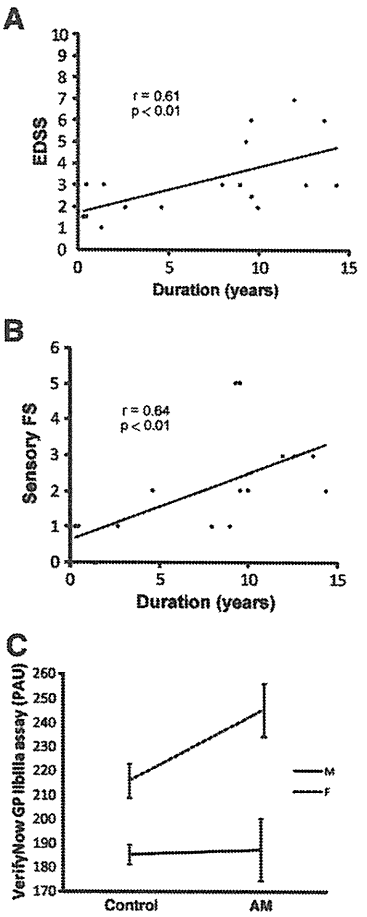

Fig. 1. (A) Correlation between the disease duration and the EDSS scores in the AM patients. (B) Correlation between the disease duration and the Sensory FS scores in the AM patients. (C) Two-way ANOVA of the GP IIb/IIIa values by sex and disease. The GP IIb/IIIa levels are significantly higher in females than in males and in the AM patients compared with the controls. AM: atopic myelitis; EDSS: Expanded Disability Status Scale of Kurtzke's score; FS: Functional Scale; GP IIb/IIIa: glycoprotein IIb/IIIa.

Table 1
Comparisons of the hematological and allergological findings between the AM patients and healthy controls.

	AM patients ($n=17$)	Healthy controls ($n=35$)	p value[a]
Bronchial asthma	7 (41.2%)	1 (2.9%)	<0.001
Atopic dermatitis	5 (29.4%)	5 (14.3%)	NS
Allergic rhinitis	10 (58.8%)	8 (22.9%)	<0.05
Food allergy	6 (35.3%)	3 (8.6%)	<0.05
Allergic conjunctivitis	5 (29.4%)	2 (5.7%)	<0.05
White blood cells (/μl)	7174.7 ± 2212.1	6122.9 ± 967.7	NS
Neutrophils (/μl)	4570.7 ± 1881.7	3417.5 ± 819.4	<0.05
Lymphocytes (/μl)	2010.2 ± 747.1	2088.3 ± 358.4	NS
Eosinophils (/μl)	235.0 ± 173.6	156.7 ± 129.8	NS
Platelets (× 10,000/μl)	24.8 ± 5.3	23.3 ± 6.4	NS
Hemoglobin (g/dl)	13.0 ± 1.5	13.9 ± 1.5	NS
Total IgE (IU/ml)	996.5 ± 1619.2	143.2 ± 188.0	<0.05
Allergen specific IgE to *Dermatophagoides pteronyssinus* (UA/ml)	24.6 ± 37.0	9.4 ± 15.4	NS
Allergen specific IgE to *Dermatophagoides farinae* (UA/ml)	23.6 ± 34.6	8.1 ± 13.3	NS

Data are shown as means ± SD.
AM: atopic myelitis; NS: not significant.

significantly higher in males than in females (14.9 ± 1.1 vs. 12.7 ± 1.1 g/dl, $p<0.01$ for all subjects).

3.3. GP IIb/IIIa values and their relationships with clinical parameters

The GP IIb/IIIa values tended to be higher in the AM patients (mean ± SD: 224.8 ± 44.1) than in the healthy controls (201.9 ± 29.5) as a whole group ($p=0.06$). Considering the sex differences as a secondary factor affecting the GP IIb/IIIa differences between the two groups, we performed a two-way ANOVA for further analysis (Fig. 1 C). The two-way ANOVA of the GP IIb/IIIa values by sex and disease revealed significant main effects for sex ($F[1,51]=22.56$, $p<0.01$) and disease ($F[1,51]=4.69$, $p<0.05$). There was no sex-by-disease interaction. Thus, the GP IIb/IIIa values were significantly higher in females than in males in both the AM patients and controls, and were also significantly greater in the AM patients than in the controls.

3.4. Correlations between the GP IIb/IIIa values and clinical parameters

In the AM patients, the GP IIb/IIIa values showed a significant positive correlation with the platelet counts ($r=0.57$, $p<0.05$) (Fig. 2A). In contrast, there was no correlation between the GP IIb/IIIa values and the platelet counts in the controls (Fig. 2A). In the AM patients, there was a significant positive correlation between the platelet counts and eosinophil counts ($r=0.49$, $p<0.05$). In contrast, the platelet counts in the controls had a negative correlation with the eosinophil counts ($r=-0.52$, $p<0.01$) (Fig. 2B). In addition, the platelet counts showed significant positive correlations with both the *Dp* ($r=0.58$, $p<0.05$) and *Df* ($r=0.61$, $p<0.05$) levels (Fig. 2 C). Meanwhile, the GP IIb/IIIa values had a tendency to show a mild negative correlation with the hemoglobin concentrations ($r=-0.48$, $p=0.05$) in the AM patients, while there was a significant negative correlation between the GP IIb/IIIa values and the hemoglobin concentrations in the controls ($r=-0.64$, $p<0.01$) (Fig. 2D). No correlations of the GP IIb/IIIa values were found with the other clinical and laboratory parameters, including age at onset, age at examination, EDSS scores, Pyramidal FS scores, Sensory FS scores, disease duration, Progression Indexes, white blood cell counts, eosinophil counts, neutrophil counts, and total and allergen-specific IgE levels.

4. Discussion

The main new findings of the present study are as follows: (1) in AM patients, the disease duration had significant positive correlations with the EDSS scores and Sensory FS scores, but not the Pyramidal FS scores; (2) the GP IIb/IIIa values were significantly higher in the AM patients than in the controls, as well as in females compared with males; (3) the GP IIb/IIIa levels showed a significant positive correlation with the platelet counts in the AM patients, but not in the controls; and (4) the platelet counts in the AM patients showed significant positive correlations with the eosinophil counts and mite antigen-specific IgE levels.

AM patients predominantly present a fluctuating course of paresthesia/dysesthesia in the distal parts of all four limbs (Osoegawa et al., 2003a; Isobe et al., 2009). The present study has revealed for the first time a positive correlation of the disease duration with the EDSS scores in AM patients, suggesting that AM is essentially a progressive disease in most patients, although superimposed fluctuations of the symptoms may occur (Osoegawa et al., 2003a; Isobe et al., 2009). The disease preferentially affects the posterior column of the spinal cord radiologically as well as pathologically, which is in accord with the positive correlation of the disease duration with the Sensory FS scores but not the Pyramidal FS scores. Thus, the disability of AM patients over the clinical course is considered to be determined by the posterior column sensory impairment.

The GP IIb/IIIa values had a significant negative correlation with the hemoglobin levels in the controls and showed a tendency toward a negative correlation with the hemoglobin levels in the AM patients. This may be explained by the methodological reason that the VerifyNow system is a kind of turbidity assay, which leads us to a cautious interpretation of the results. The lower GP IIb/IIIa levels in males compared with females may partly reflect the higher hemoglobin levels in males than in females, because higher hemoglobin amounts reduce the absorbance, thereby lowering the GP IIb/IIIa levels in the present assay. However, Faraday et al. (1997) reported that a higher proportion of GP IIb/IIIa was activated in females compared with males, suggesting that the elevated GP IIb/IIIa levels in females may represent a physiological sex difference in platelet activity. In the present study, however, the hemoglobin levels did not differ significantly between the AM patients and controls. Furthermore, although the age at examination was higher in the AM patients than in the controls, the GP IIb/IIIa values had no correlation with the age at examination. Thus, the elevated GP IIb/IIIa levels in the AM patients are supposed to be real rather than artifacts.

Activated GP IIb/IIIa binds to fibrinogen or von Willebrand factor, thereby forming molecular bridges between aggregating platelets, and an increased amount of GP IIb/IIIa is associated with a higher platelet aggregation function (Yakushkin et al., 2011). Therefore, the increased GP IIb/IIIa amounts in the AM patients suggest a possible exaggerated reactivity of platelets in this condition *in vivo*. Atopy-related neural disorders, in which microcirculatory disturbance is

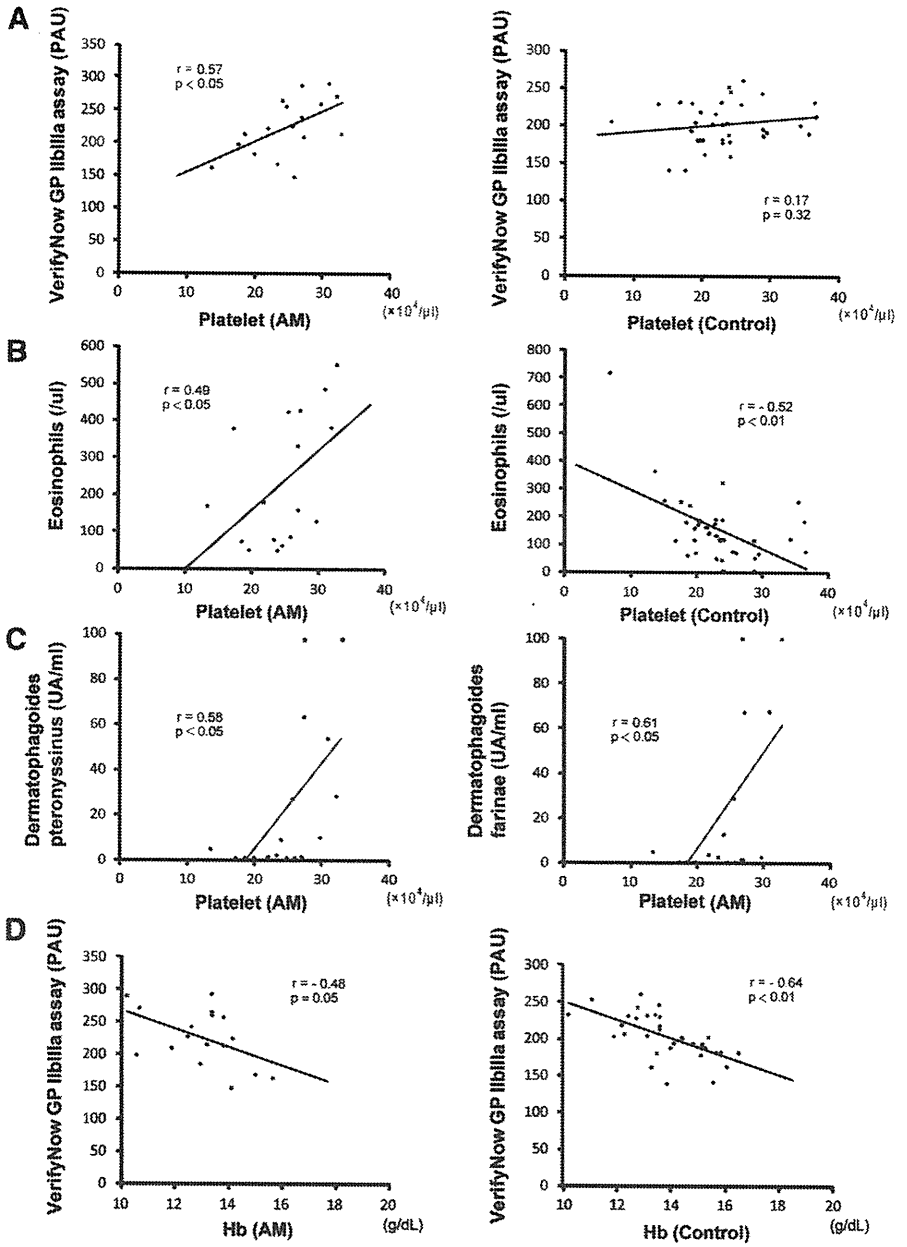

Fig. 2. (A) Correlation between the GP IIb/IIIa values and the platelet counts in the AM patients and controls. (B) Correlation between the eosinophil counts and the platelet counts in the AM patients and controls. (C) Correlation between the *Dp*- and *Df*-specific IgE levels and the platelet counts. (D) Correlation between the GP IIb/IIIa values and the hemoglobin concentrations in the AM patients and controls. GP IIb/IIIa: glycoprotein IIb/IIIa; AM: atopic myelitis; *Df*: *Dermatophagoides farinae*; *Dp*: *Dermatophagoides pteronyssinus*.

assumed, are not only limited to CSS, but may also exist in several other neurological conditions associated with atopic diathesis, such as juvenile muscular atrophy of the distal upper limb (Hiramaya disease) (Hirayama et al., 1959) and Hopkins syndrome (Hopkins, 1974). We (Kira and Ochi, 2001) and others (Ito et al., 2005) reported an association of atopic diathesis with Hirayama disease, in which shrinkage and necrosis of the anterior horns of the cervical spinal cord were noted at autopsy (Hirayama et al., 1987). Another rare disease is an acute poliomyelitis-like illness known as Hopkins syndrome (asthmatic amyotrophy). The disease presents as sudden onset of flaccid paralysis following asthma attacks in children (Ito et al., 2005), and responds poorly to corticosteroids in most cases (Shahar et al., 1991). We also reported cases of AM showing focal amyotrophy and anterior horn cell involvement (Tokunaga et al., 2004; Kira et al., 2008), suggesting possible links of AM with Hopkins syndrome and Hirayama disease (Kira et al., 2008). In Hirayama disease, repeated microcirculatory disturbances are assumed to cause anterior horn cell necrosis, which is vulnerable to ischemia (Hirayama, 2000).

It has been shown that intravascular platelet activation is necessary for the development of chronic airway inflammation (Kowal et al., 2006; Pitchford and Page, 2006). In the acute phase of asthma attacks, not only eosinophils but also platelet activation markers, such as β-thromboglobulin, platelet factor-4 and soluble P-selectin, are elevated during allergen challenge with *Dp* (Kowal et al., 2006). It was reported that eosinophils from allergic patients showed enhanced interactions with platelets, and that P-selection on platelets bound to eosinophils reinforced the tethering of these cells to endothelia, thereby potentiating the migration of eosinophils into the parenchyma (Ulfman et al., 2003). The significant positive correlations

of the platelet counts with the eosinophil counts and *Dp*- and *Df*-specific IgE levels in AM patients may suggest a possible positive interaction of these factors. It is possible that elevated mite antigen-specific IgE may potentiate the migration of increased numbers of eosinophils into the inflamed spinal cord through eosinophil/platelet interactions, thereby contributing to the tissue damage in AM patients.

In the present study, we have revealed that AM is a progressive disease and that the platelet aggregative function is increased in AM. Thus, long-term use of an anti-platelet agent may be worth trying to prevent disease progression in this condition.

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cognitive decline.[6] Furthermore, the *APOE* ε4 allele was associated with greater decreases in $A\beta_{42}$ and increases in t-tau. Another study found stability of CSF $A\beta_{42}$ and t-tau levels among cognitively normal subjects, persons with MCI, and persons with AD over a 1-year period except for a modest increase in t-tau in elderly controls (mean age 75).[7]

Our findings indicate that CSF biomarker changes occur early during the presymptomatic state in familial AD and we found substantial changes between 22 and 17 years before the expected onset of dementia. Though persons carrying familial AD mutations allow one to sensitively identify the time course of biomarker changes during the presymptomatic period, the degree to which these findings can be generalized to late-onset AD is unclear. Verification of our results with larger numbers of subjects awaits larger studies such as those of the Dominantly Inherited Alzheimer Network (http://www.dian-info.org/).

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INTERLEUKIN-7 RECEPTOR ALPHA GENE POLYMORPHISM INFLUENCES MULTIPLE SCLEROSIS RISK IN ASIANS

A recent genome-wide survey identified non–human leukocyte antigen (*HLA*) genes that are related to multiple sclerosis (MS). Among these, an association of a single nucleotide polymorphism (SNP), rs6897932, in the interleukin-7 receptor α gene (*IL-7RA*) with MS susceptibility has been widely replicated in Caucasians.[1–3] The SNP located in the transmembrane domain of IL-7Rα is nonsynonymous and functional: the MS-susceptible CC allele increases levels of the soluble form of IL-7Rα via exon skipping, and decreases the expression of membrane-bound IL-7Rα, thereby causing decreased IL-7/IL-7R signaling.[1–3] IL-7/IL-7R signaling induces thymic production of $FOXP3^+$ regulatory T cells, which efficiently ameliorate experimental autoimmune encephalomyelitis,[4] an animal model of MS. Thus, the rs6897932 polymorphism of the IL-7RA gene may confer MS susceptibility through decreased production of $FOXP3^+$ regulatory T cells due to downregulated IL-7/IL-7R signaling. This polymorphism has never been reported in either MS or neuromyelitis optica (NMO) in Asians. Therefore, in the present cross-sectional study, we investigated the association of the IL-7RA SNP rs6897932 with non-NMO MS and NMO in the Japanese.

Methods. All patients with NMO fulfilled the 2006 Wingerchuk[5] criteria for NMO, while those with NMO spectrum disorders who did not completely meet the criteria were excluded. All non-NMO patients with MS satisfied the revised McDonald criteria for MS[6] but did not meet the Wingerchuk criteria for NMO. All patients were thoroughly examined in the Neurology Departments of the University Hos-

pitals of the South Japan MS Genetics Consortium (Coinvestigators). Samples from 265 patients and 158 unrelated healthy controls (HCs) were collected between 1987 and 2010. Among the 265 patients, 73 were positive for an anti-aquaporin-4 (AQP4) antibody while 192 were negative. Sixty-five patients positive for anti-AQP4 antibody and 13 patients negative for the antibody met the NMO criteria[5] and were regarded as patients with NMO. Additionally, the present patients, excluding 20 patients with primary progressive MS, were also classified into 107 conventional MS (CMS) and 138 opticospinal MS (OSMS) including spinal and brainstem-spinal MS.[7]

The SNP rs6897932 in the IL-7RA gene was genotyped by real-time PCR using TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA). The genotype of each sample was defined automatically by allele-specific fluorescence, using an ABI Prism 7500 sequence detection system (Applied Biosystems). The test for Hardy-Weinberg equilibrium demonstrated that the HCs and cases of all the other subgroups were in allelic population equilibrium. The allele and genotype frequencies among the groups were compared using Fisher exact test. Uncorrected p values were corrected by Bonferroni-Dunn correction to calculate corrected p (p_{corr}) values.

Standard protocol approvals, registrations, and patient consents. This study was approved by each institutional ethics committee. Informed consent was obtained from all participants.

Results and discussion. The frequencies of both the C allele and the CC genotype of SNP rs6897932 in the *IL-7RA* gene in patients with non-NMO MS were significantly higher than those of HCs (table). However, there was no significant difference in the frequency of either the C allele or the CC genotype between HCs and patients with NMO. The frequencies of both the C allele and the CC genotype were significantly higher in patients with CMS than in HCs (90.65% vs 79.75%, $p_{corr} = 0.0020$, odds ratio [OR] = 2.46, 95% confidence interval [CI] 1.44–4.21, and 81.31% vs 63.29%, $p_{corr} = 0.0048$, OR = 2.52, 95% CI 1.41–4.52, respectively), but not in patients with OSMS (87.68% vs 79.75%, and 75.30% vs 63.29%, respectively, $p_{corr} > 0.05$).

This study revealed a significant association of the SNP rs6897932 of *IL-7RA* gene with non-NMO MS in Japanese populations. In a case-control study conducted by the International Multiple Sclerosis Genetics Consortium among white populations, the C allele of rs6897932 was shown to be a susceptibility allele for MS, with an OR of 1.18 (1.11–1.26),[3] while a North European group reported similar findings; the OR for the C allele was 1.32 (1.11–1.54).[2] By contrast, our OR for the C allele in non-NMO MS was much higher than those in the abovementioned studies, although the number of participants in this study was small. We assume that this allele is a much stronger risk factor for non-NMO MS in Asians than in Caucasians. Moreover, since in the present series we found no significant difference in either allele or phenotypic frequency of *HLA-DRB1*1501* between non-NMO MS and HC (12.6% vs 8.9% and 23.5% vs 17.7%, respectively, $p_{corr} > 0.05$), we suggest that in Asians, the effect of rs6897932 in IL-7RA is greater than that of *HLA-DRB1*1501*, which is the strongest MS susceptibility gene allele in Caucasians. Finally, because of the rar-

Table **Allele and genotype frequencies for *IL-7RA* SNP rs6897932 among patients with NMO, non-NMO MS, and healthy controls**

			NMO vs HC, C/T[a]			Non-NMO MS vs HC, C/T[a]	
	HC (n = 158), n (%)	NMO (n = 78), n (%)	p_{corr}	OR (95% CI)	Non-NMO MS (n = 187), n (%)	p_{corr}	OR (95% CI)
Allele frequencies							
T allele	64 (20.25)	20 (12.82)	0.1644	1.73 (1.00–2.97)	40 (10.70)	0.0018[b]	2.12 (1.38–3.25)
C allele	252 (79.75)	136 (87.18)			334 (89.30)		
			NMO vs HC, CC/TT+TC[c]			*Non-NMO MS vs HC, CC/TT+TC*[c]	
Genotype frequencies							
TT	6 (3.80)	0 (0.00)	0.3180	1.68 (0.92–3.07)	0 (0.00)	0.0056[b]	2.13 (1.32–3.43)
TC	52 (32.91)	20 (25.64)			40 (21.39)		
CC	100 (63.29)	58 (74.36)			147 (78.61)		

Abbreviations: CI = confidence interval; HC = healthy controls; *IL-7RA* = interleukin-7 receptor α gene; MS = multiple sclerosis; NMO = neuromyelitis optica; OR = odds ratio; p_{corr} = corrected p value; SNP = single nucleotide polymorphism.
[a] χ^2 Test was used to see the effect of C allele vs T allele.
[b] Significant.
[c] Fisher exact test was used to assess the statistical significance of CC vs TT+TC (recessive model for C).

ity of NMO, its sample size was not large in the present study and larger cohort studies are required to confirm our findings.

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ORIGINAL ARTICLE

SNP-based analysis of the HLA locus in Japanese multiple sclerosis patients

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*Although several major histocompatibility complex (MHC)-wide single-nucleotide polymorphism (SNP) studies have been performed in populations of European descent, none have been performed in Asian populations. The objective of this study was to identify human leukocyte antigen (HLA) loci associated with multiple sclerosis (MS) in a Japanese population genotyped for 3534 MHC region SNPs. Using a logistic regression model, two SNPs (MHC Class III SNP rs422951 in the NOTCH4 gene and MHC Class II SNP rs3997849, susceptible alleles A and G, respectively) were independently associated with MS susceptibility (204 patients; 280 controls), two (MHC Class II SNP rs660895 and MHC Class I SNP rs2269704 in the NRM gene, susceptible alleles G and G, respectively) with aquaporin-4− (AQP4−) MS susceptibility (149 patients; 280 controls) and a single SNP (MHC Class II SNP rs1694112, susceptible allele G) was significant when contrasting AQP4+ against AQP4− patients. Haplotype analysis revealed a large susceptible association, likely DRB1*04 or a locus included in the DRB1*04 haplotype, with AQP4− MS, which excluded DRB1*15:01. This study is the largest study of the HLA's contribution to MS in Japanese individuals.*
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Keywords: *multiple sclerosis; HLA; Japanese; MHC; AQP4*

Introduction

Multiple sclerosis (MS) is the prototypic disease of central nervous system (CNS) myelin and is considered to be autoimmune in origin. Although the exact cause of MS is unknown, there is an unequivocal, if partial, genetic contribution to its pathogenesis.[1,2] Although several loci with modest replicable effects on MS susceptibility have been identified (mostly in studies of populations of European descent), the only genetic region identified with a large, consistently replicated effect in all populations is the human leukocyte antigen (HLA) region on chromosome 6p21.3. The primary signal arises from the *HLA-DRB1* gene in the Class II segment of the locus, more specifically the **15:01* allele of *DRB1*, but complex hierarchical allelic effects, copy number and *cis–trans* regulatory interactions across the entire region, including a protective signal in the Class I region, have been reported as well.[3–9]

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MS is relatively rare in Asians, but clinical heterogeneity is worth noting. Some have a disease, termed conventional MS (CMS), which is indistinguishable from MS in western countries (disseminated lesions in the CNS), whereas others have a variant, termed opticospinal MS (OSMS), which involves predominantly the optic nerve and spinal cord.[10] The exact relationship between CMS and OSMS is uncertain; OSMS might represent a true variant of CMS or a phenocopy that is biologically unrelated to CMS. HLA data suggest that the two forms are immunogenetically distinct. In studies of the HLA in Japanese MS populations, CMS was associated with *HLA-DRB1*15:01*,[10] whereas OSMS was associated with the centromeric *HLA-DPB1* locus,[10–12] both Class II major histocompatibility complex (MHC) genes. Recently, autoantibodies against the cell membrane water channel aquaporin-4 (AQP4), a specific biomarker for neuromyelitis optica (NMO), were identified in a proportion of patients with OSMS, leading to a reclassification of this entity based on seropositivity to AQP4. Because the distinction between MS and NMO (an inflammatory disease affecting only the optic nerves and spinal cord) and their etiologies is not clear, especially in the Japanese population where there is a higher relative prevalence of OSMS,[13] a well-defined biomarker such as AQP4 seropositivity may be more useful for stratification

for genetic analyses. Indeed, in Japanese populations, *HLA-DRB1*12* was found to be a risk factor for anti-AQP4 antibody-positive patients, but not antibody-negative MS.[14] However, a comprehensive investigation of the entire HLA region in Japanese MS has yet to be performed.

The advent of large-scale single-nucleotide polymorphism (SNP)-based genotyping allowed broad analyses of the HLA region in MS. For example, several whole-genome association studies have been completed in individuals of European descent,[15–23] which included thousands of SNPs in the HLA region. Most of these studies validated the strong Class II *HLA-DRB1*15:01* association with disease susceptibility, as well as HLA Class I[20,24] and a *DRB1*03:01-DQB1*02:01* (ref. 23) associations. In addition, a few studies have focused exclusively on SNPs in the HLA region using customized arrays, confirming the presence of a disease locus in the Class I region.[6,9,24] To date, significant Class I associations have not been identified in Japanese MS. The unique patterns of linkage disequilibrium (LD) between different ethnic groups have represented a powerful means to delineate causative disease-associated variants in the HLA region.[25–27] The objective of this study is to identify HLA loci associated with MS in a Japanese data set using a high-density SNP array. In addition, because of the complexities of defining disease subclasses and the resulting sizes of those subclasses in this population, a biomarker (presence or absence of autoantibodies against AQP-4) was used instead to stratify the population for further refinement of genetic associations.

Results

Population parameters

A total of 280 healthy controls (HC) and 204 individuals with MS with data for 3534 HLA region SNPs remained after all quality control (QC) steps (Table 1). In all, 46% of the HC and 75% of the individuals with MS were women (gender was included as a covariate for all analyses). A total of 55 of all patients were positive for anti-AQP4 antibody (AQP4+). Among 193 cases that fulfill the revised McDonald criteria,[28] 51 cases (26.4%) are positive for the anti-AQP4 antibody.

Table 1 Clinical phenotypes

	Anti-AQP4Ab+ (n=*55*)		*Anti-AQP4Ab–* (n=*149*)	
	NMO[a]+ (n=*38*)	*NMO–* (*n*=*17*)	*NMO+* (*n*=*8*)	*NMO–* (*n*=*141*)
McDonald criteria[b] (+) (*n*=193)	35	16	8	134
CMS (*n*=110)	9	9	1	91
OSMS (*n*=52)	25	3	7	17
Others[c] (*n*=31)	1	4	0	26
McDonald criteria (–) and criteria for CIS[d] (+) (*n*=11)	3	1	0	7

Abbreviations: Ab, antibody; AQP4, aquaporin-4; CIS, clinically isolated syndrdome; CMS, conventional multiple sclerosis; NMO, neuromyelitis optica; OSMS, opticospinal multiple sclerosis.
[a]The revised criteria for NMO by Wingerchuk *et al.*[50]
[b]The revised McDonald criteria by Polman *et al.*[28]
[c]Those cases who fulfill the revised McDonald criteria, but did not meet the criteria for CMS or OSMS.
[d]CIS criteria by Dalton *et al.*[41]

Multidimensional scaling was used to determine the ethnic relationship between the Japanese MS cohort and 12 HapMap populations. By plotting the first by the second dimension, all patients and controls cluster with the HapMap Japanese and Chinese populations (Supplementary Figure 1), verifying that they are indeed East Asian. Plotting all informative dimensions ($n=7$) separately, patients and controls always cluster with the HapMap Japanese population, including dimension 7, which separates the Chinese from the Japanese populations (Figure 1), verifying that the experimental sample is non-Chinese East Asian (genotyping data for other East Asian populations was not available).

Association analyses

The two SNPs typically used to infer *DRB1*15* status (rs3135388 and rs9271366 (ref. 29)) were not useful for determining **15:01* carrier status and copy number in this data set. The SNP rs3135388 is fixed for the G allele in the current population, as well as the HapMap Japanese population, and rs9271366 captured in our data set both the **15:01* and **15:02* alleles. The association between rs9271366 and all MS and AQP4– MS was not significant (comparison-wise $P=0.33$ and 0.15, respectively). Across the (classical) HLA-typed individuals, 48% of the **15* alleles in the data set were **15:02*, indicating that the **15:02* alleles are likely diluting the risk effect of the **15:01* alleles for this particular SNP. Therefore, only *DRB1*15:01* presence or absence data, from *DRB1*15:01*-specific primers,[25] was used for the experimental sample and was moderately associated with MS ($P=0.014$) and AQP4– MS ($P=0.01$); however, this association was modest compared with the SNP associations (see below: trend test, $P=10^{-6}$–10^{-7}).

Two SNPs were associated with MS using the iterative model (Table 2). Rs422951 in the Class III region had the most significant association with MS ($P=2.9\times10^{-6}$; odds ratio (OR)=0.4; 32 296 360 bp) (Supplementary Figure 2A). This SNP results in a missense mutation in *NOTCH4*. After fitting rs422951 into the model, rs3997849 was the most significant SNP ($P=8.1\times10^{-5}$; OR=0.5; 32 790 379 bp). The SNP rs3997849 is in the HLA Class II region, closest to the *HLA-DQA2* gene (26 761 bp away). When fitting both SNPs as covariates in the model, no other SNPs were significant for MS at false discovery rate (FDR) $P<0.1$.

Two SNPs were significant for AQP4– patients vs controls using the iterative model (Table 2). SNP rs660895 was significant ($P=6.2\times10^{-7}$; OR=2.23; 32 685 357 bp) with no SNPs in the model (Supplementary Figure 2B). This SNP is in the Class II region and is closest to the *HLA-DRB1* and *HLA-DRB5* genes (19 817 bp away). After fitting rs660895 into the model, rs2269704 was the most significant SNP ($P=1.64\times10^{-4}$; OR=0.27; 30 764 931 bp). This SNP is in the Class I region and is in an intron of the *NRM29/NRM* gene. After fitting these two SNPs, no other SNPs were significant for AQP4– MS susceptibility at FDR $P<0.1$.

Only a single SNP, rs1694112, was significant ($P=1.34\times10^{-5}$; OR=3.30; 32 757 641 bp) when contrasting AQP4– vs AQP4+ patients (Supplementary Figure 2C). This SNP is in the Class II region and is closest to

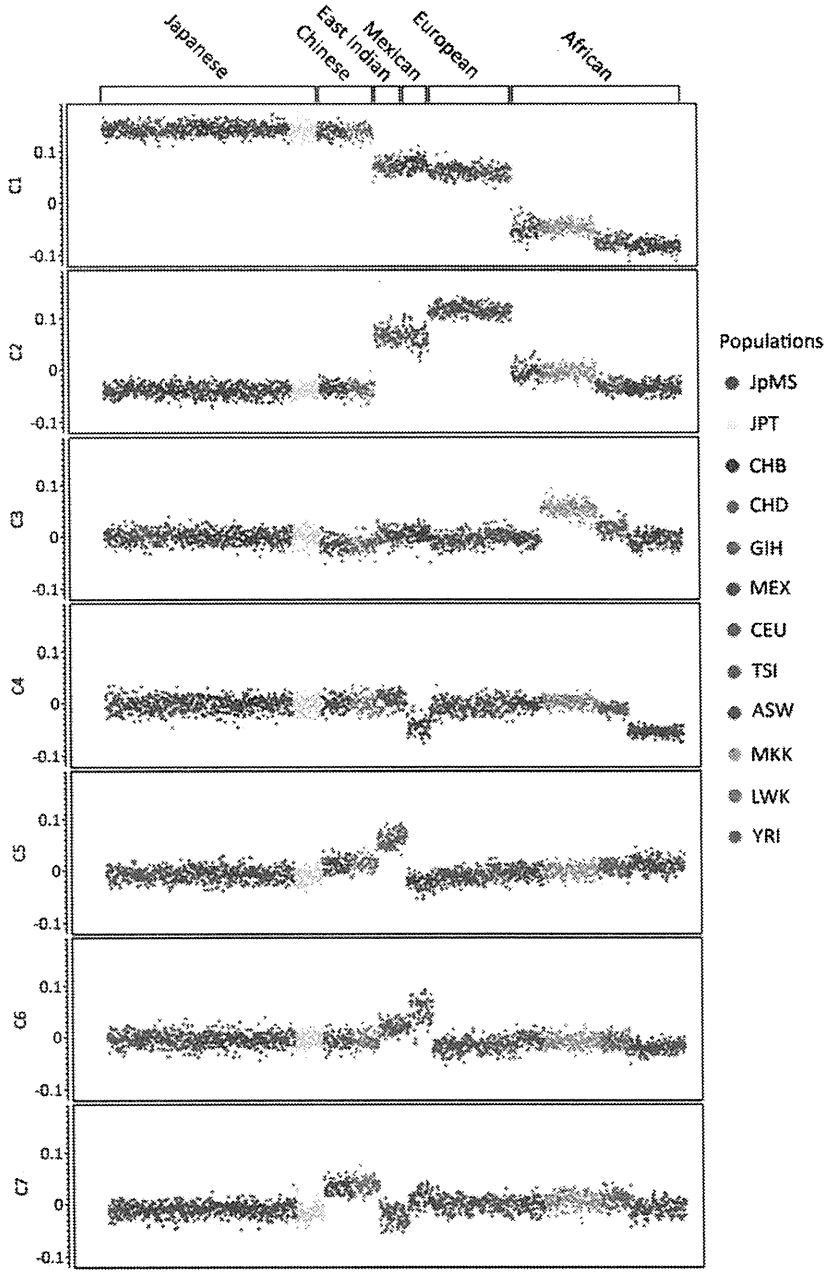

Figure 1 Plot of each of the first seven multidimensional scaling dimensions individually. The Japanese MS (JpMS) sample always clusters with the HapMap Japanese population, including dimension 7, which separates the Chinese from the Japanese populations. The *y* axis is the value for each of the first seven dimensions. ASW, African ancestry in Southwest USA; CEU, CEPH (NW European); CHB, Han Chinese in Beijing, China; CHD, Chinese in Denver, Colorado; GIH, Gujarati Indians in Houston, Texas; JPT, Japanese in Tokyo, Japan; LWK, Luhya in Webuye, Kenya; MEX, Mexican ancestry in Los Angeles, California; MKK, Maasai in Kinyawa, Kenya; TSI, Tuscan in Italy; YRI, Yoruban in Ibadan, Nigeria.

the *HLA-DQB1* gene (15 197 bp away). No SNPs were significant (FDR $P>0.1$) when contrasting AQP4+ patients vs controls.

Haplotype analyses

Haplotype analyses were performed to further clarify the roles of the significant SNPs in tagging causative variation (Table 3). For MS, using a haplotype dosage model, three Class II–class III haplotypes (rs422951–rs3997849: G–G, G–A, A–A) were at least suggestively (comparison-wise $P<0.1$) associated in the resistant direction (OR <1), and the remaining haplotype (rs422951–rs3997849: A–G) was significantly associated in the susceptible direction. All three 'resistant' haplotypes are significantly associated with resistance when fit together in the multivariate model. For AQP4– demyelinating patients vs controls, two haplotypes (rs660895–rs2269704: A–G, A–A) were significantly associated in the resistant direction, whereas a single haplotype (rs660895–rs2269704: G–G) was associated

Table 2 SNPs significantly (FDR $P = 0.1$) associated with MS in the Japanese sample

Comparison	N *SNPs in model*	*SNP*	*Raw P-value*	*FDR P*	*Odds ratio*	*Susceptible allele*	*Position*	*Class*	*Closest gene*	*Distance to closest gene (bp)*
MS vs cont.	0	rs422951	2.94E−06	6.08E−03	0.4	A	32 296 360	III	*NOTCH4*	0
MS vs cont.	1	rs3997849	8.07E−05	5.59E−02	0.5	G	32 790 379	II	*HLA-DQA2*	26 761
AQP4− vs cont.	0	rs660895	6.23E−07	6.61E−04	2.23	G	32 685 357	II	*HLA-DRB1, HLA-DRB5*	19 817
AQP4− vs cont.	1	rs2269704	1.64E−04	8.49E−02	0.27	G	30 764 931	I	*NRM29,NRM*	0
AQP4+ vs AQP4−	0	rs1694112	1.34E−05	4.74E−02	3.3	G	32 757 641	II	*HLA-DQB1*	15 197

Abbreviations: AQP, aquaporin-4; FDR, false discovery rate; LD, linkage disequilibrium; MS, multiple sclerosis; SNP, single-nucleotide polymorphism.
The most significant SNP from each iteration was fit as a covariate for the remaining iterations (*N* SNPs in Model) to remove associations resulting from LD with that SNP. Gender was an additional covariate in all iterations. Minor allele is reference for odds ratios. MS vs Cont = all patients ($n = 204$) vs controls ($n = 280$); AQP4− vs controls = AQP4− patients ($n = 149$) vs controls ($n = 280$); AQP4+ vs AQP4− = AQP4+ patients ($n = 55$) vs AQP4− patients ($n = 149$).

Table 3 Haplotype associations with MS susceptibility

Comparison	*SNPs*	*Haplotype*	*OR*	P-*value*	*Frequency*
(a) *Individual haplotype dosage*					
MS vs controls	rs422951–rs3997849	GG	0.41	3.46E−06	0.18
MS vs controls	rs422951–rs3997849	AG	2.45	5.17E−10	0.6
MS vs controls	rs422951–rs3997849	GA	0.33	8.46E−02	0.01
MS vs controls	rs422951–rs3997849	AA	0.62	5.18E−03	0.21
AQP4− vs controls	rs660895–rs2269704	GG	2.32	9.04E−08	0.3
AQP4− vs controls	rs660895–rs2269704	AG	0.71	2.12E−02	0.6
AQP4− vs controls	rs660895–rs2269704	GA	0.43	2.76E−01	0.01
AQP4− vs controls	rs660895–rs2269704	AA	0.22	1.93E−06	0.09

Comparison	*SNPs*	*Haplotype*	*OR*	P-*value*
(b) *Multivariate model*				
MS vs controls	rs422951–rs3997849	GG	0.33	4.01E−08
MS vs controls	rs422951–rs3997849	GA	0.25	3.06E−02
MS vs controls	rs422951–rs3997849	AA	0.49	4.49E−05
AQP4− vs controls	rs660895–rs2269704	AG	0.5	1.22E−05
AQP4− vs controls	rs660895–rs2269704	AA	0.13	1.54E−09

Abbreviations: AQP, aquaporin-4; MS, multiple sclerosis; OR, odds ratio; SNP, single-nucleotide polymorphism.
Haplotypes formed from the two SNPs significant for each for MS and AQP4− MS. (a) Association of dosage of each haplotype with MS susceptibility (MS vs controls) or AQP4− MS susceptibility (AQP4− vs controls). (b) Multivariate model with all three susceptible haplotypes for all MS and both significant susceptible haplotypes for AQP4− MS. Gender was fit as a covariate in all models.

with susceptibility. Both resistant haplotypes were highly significant when fit in the multivariate model.

Haplotype tagging of DRB1 *alleles*

As the *HLA-DRB1* gene, and specifically the **15:01* allele, has been shown in populations of European descent and in Japanese to have an association with MS susceptibility, the two-locus haplotypes associated with MS and the single SNP associated with AQP4− vs AQP4+ patients were scrutinized for their ability to tag *DRB1* alleles in the subset of individuals ($n = 218$) with *DRB1* data (Supplementary Table 1; Supplementary Figure 3). For the MS group, the most significantly associated and only susceptible haplotype was AG (rs422951–rs3997849). This haplotype captures 100% of the *DRB1*15:01* alleles, which may explain part of the association with MS, but also captures most of the **08:03*, **08:02* and **04* alleles. The association of this haplotype with MS could be due to any single allele or combinations of these *DRB1* alleles. Interestingly, the 53% of the A–A resistant haplotypes contain *DRB1*15:02* alleles, and all of the **15:02* alleles are captured by this haplotype. The GG haplotype, which is the most associated resistant haplotype, is fairly evenly split between three *DRB1* alleles: **01:01*, **13:02* and **09:01*. The G–A haplotype is at such a low frequency in the population (1.4%) that interpretations of *DRB1* allele tagging for this haplotype are likely not meaningful.

For the AQP4− vs HC comparisons, the most significantly associated and only susceptible haplotype was GG (rs660895–rs2269704). Interestingly, this haplotype excludes *DRB1*15:01*, but includes all of the **08:02* and **12:02* alleles and most of the **04* alleles. The most associated resistant haplotype (A–A) mostly contains

13:02 alleles (sensitivity = 75%; positive predictive value = 75%). The A–G haplotype, which is mildly associated with resistance, captures all of the **15:01* and **15:02* alleles, and the G–A haplotype is too rare to make sound conclusions. Finally, the SNP associated with AQP4− vs AQP4+ does not capture any single *DRB1* allele well enough to draw *DRB1*-specific conclusions.

Discussion

The HLA region has repeatedly shown a strong association with MS in studies of individuals of European descent.[2] The *HLA-DRB1*15:01* allele, as observed through classical HLA typing and **15:01* tagging SNP studies, is the likely source of the major HLA effect in individuals of European descent. In the Japanese population, which has a higher frequency of OSMS than populations of European descent, the **15:01* allele was found to be associated with CMS only.[10] This study investigated the association between 6040 HLA region SNPs with MS in 204 Japanese patients and 280 Japanese controls.

A total of 2506 SNPs were removed from the analysis owing to QC. The majority (65%) of these SNPs were removed because of minor allele frequency < 0.05. This is not surprising considering that most known SNPs were discovered in non-Asian populations, and that there is less genetic variation in the Japanese population as compared with many of populations in which the SNPs were identified. The total number of SNPs passing QC and remaining in the analysis was 3534. The reduction in the total number of SNPs from QC decreased the coverage of the MHC region to an average of 707 SNPs per Mb.

Although the number of individuals were modest for an association study ($n = 280$, 204, 149 and 55 for controls, all patients, AQP4− and AQP4+ patients, respectively), this study is the largest of its kind in Japanese MS. For the main analysis with 204 patients, the power, calculated using the Power for Genetic Association Analyses program[30] (co-dominant model, disease prevalence = 0.001, disease allele frequency = 0.2, marker allele frequency = 0.2, effective degrees of freedom = 3429 (calculated using the EDF program included with Power for Genetic Association Analyses), $\alpha = 0.1$), was good to detect large effects (power = 0.97 for relative risk = 3), but not moderate effects (power = 0.4 for relative risk = 2). Power was considerably less for the other analyses with fewer patients, as expected (data not shown). Consequently, as in any study of the genetics of a complex disease, this study likely does not capture all biologically associated loci or loci with small effects if they are present. The results and discussion herein therefore pertain to those effects that the study had the power to identify.

Fitting the most significant SNP from an MHC-wide analysis as a covariate in subsequent MHC-wide analyses identified two SNPs that were significantly (FDR $P = 0.1$) associated with MS. It was decided *a priori* to use an FDR $P = 0.1$ to strike a balance between power and false negatives. At this significance level, 90% of all significant associations are expected to be true positives. The sequential method allows the determination of multiple SNPs that are associated independent of the LD they share with a more significant SNP. The most significantly associated SNP (rs422951) was in the Class III region and results in a missense mutation (Thr → Ala) in the *NOTCH4* gene. *NOTCH4* is involved in cell differentiation, proliferation and apoptosis, and has been implicated as a schizophrenia-associated locus.[31] Although possible associations have been identified for other autoimmune diseases,[9] a *NOTCH4* genetic association independent of *DRB1* has not been previously shown for MS. However, the NOTCH signaling pathway may be important in T-cell activation, oligodendrocyte differentiation, remyelination and has been suggested as a target for treatment of MS.[32] Therefore, the association of the *NOTCH4* missense mutation with MS may be of great importance to identifying treatments for Japanese MS patients. The secondarily MS-associated SNP (rs3997849) is closest to the *HLA-DQA2* gene in the Class II region. This gene encodes a protein involved in antigen presentation, and although previously studied in MS in a population of European descent,[33] the gene has not been previously shown to be associated with MS independent of *DRB1*.

Classification of patients into MS or NMO disease is complex in the Japanese population. Many patients fall into both OSMS and NMO groups, and several non-NMO patients are AQP4 seropositive (Table 1). Therefore, AQP4 seropositivity, rather than disease class, was used to stratify the data for subsequent analysis. Two SNPs were significantly associated with AQP4− MS. The SNP rs660895 was the primary associated SNP, and is closest to the *DRB1* gene. *DRB1* is the strongest and most replicated associated gene with MS in populations of European descent.[2] When fitting rs660895 in the model, rs2269704, which is located in an intron of the Class I *NRM29/NRM* gene, was the most significantly associated SNP. This gene is a nuclear envelope membrane protein, and no evidence of previous associations of this gene with MS could be identified. Finally, contrasting AQP4+ and AQP4−, MS patients identified a single significantly associated SNP (rs1694112), which is closest to the *HLA-DQB1* gene.

It should be noted that both SNPs significant for all MS, rs422951 and rs3997849, were both significant for AQP4− patients vs controls (FDR $P = 0.003$ and 0.06, respectively) in the same direction, and rs660895 and rs2269704 in all MS (FDR $P = 0.006$ and 0.01, respectively). Thus, it can be concluded that the differences in top associations between the two analyses, all demyelinating and AQP4− demyelinating, is likely due to random statistical fluctuations that occur when making a small modification to a data set (removing 55 AQP4+ individuals), rather than true differences being identified by subsetting the data.

Although it is interesting, and possibly informative, to discuss the genes closest to the identified significant SNPs, the extensive and intricate LD patterns in the MHC leads to the possibility that these SNPs may be tagging a causative mutation further away than the closest genes. As *DRB1* is the classical MS-associated gene in individuals of European descent and evidence of association has been reported in Japanese MS, haplotypes formed by the SNPs were scrutinized for the ability to tag *DRB1* alleles in 218 Japanese individuals for whom four-digit *DRB1* data were available. For the analysis of