Table 1 Patient characteristics at initial presentation.

Measurements	Anti-synthetase positive (n = 13)		Anti-synthetase negative (n = 185)		p-value
Age at the onset (yr)	55.0	[44.5–60.8]	67.4	[60.6–73.2]	<0.001†
Female (n; (ratio %))	6	(46.2)	53	(28.7)	0.20
Smoking history (n)	9	(69.2)	129	(69.7)	0.78
Surgical biopsy for diagnosis (n)	8	(61.5)	36	(19.5)	<0.01†
Fever (>38 °C) (n)	2	(15.4)	10	(5.4)	0.18
Body weight loss (n)	1	(7.7)	9	(4.9)	0.50
Dyspnea (n)	12	(92.3)	154	(83.2)	0.70
Cough (n)	5	(38.5)	71	(38.4)	1.00
Other respiratory symptoms (n)[a]	1	(7.7)	9	(4.9)	0.50
Fine crackles (n)	12	(92.3)	167	(90.3)	1.00
Clubbed fingers (n)	1	(7.7)	34	(18.4)	0.47
Arthralgia or joint deformity (n)	2	(15.3)	4	(2.34)	< 0.01†
Raynaud's phenomenon (n)	1	(7.7)	4	(2.2)	0.29
Cutaneous symptoms (n)[b]	4	(30.8)	3	(1.6)	< 0.01†
Oxygen administration (n)	1	(7.7)	18	(9.7)	1.00
Treatment (n)	9	(69.2)	81	(43.8)	0.08
Corticosteroids	1	(7.7)	32	(17.2)	0.06
Corticosteroids plus immunosuppressants	8	(61.5)	49	(26.2)	
Immunosuppressants	0	(0.0)	1	(0.5)	
No medication	4	(30.8)	105	(56.1)	
Duration of the observation (months)	22.8	[17.0–35.1]	39.3	[17.0–65.0]	0.11
Survivor (n)	13	(100)	138	(74.6)	0.04*

Abbreviation: anti-synthetase = autoantibody to aminoacyl-tRNA synthetase. Numbers are expressed as median [25%–75% interquartile] or number of data (percent) Comparison was made by Fisher's exact test or chi-square test as appropriate; $p < 0.05$ *, $p < 0.01$ †.
Treatment regimens included oral corticosteroids, immunosuppressants, and intravenous pulse methylpredonisolone therapy.
[a] Other respiratory symptoms include chest pain or hemoptysis.
[b] Cutaneous symptoms include Gottron's sign and heliotrope purpura.

frequent in anti-synthetase positive cases ($p < 0.01$). However, extrapulmonary features of ASS were absent in 6 anti-synthetase positive cases (46.2%). The number of cases in treatment tended to be higher in the anti-synthetase positive group. Survival rate at the end of the observation period was higher in the anti-synthetase positive group than in the anti-synthetase negative group ($p = 0.04$); however, survival curves did not reveal a significant difference between the two groups when analyzed with the log rank test ($p = 0.22$). All the NSIP survived during the study period regardless of anti-synthetase status; thus survival differences between anti-synthetase positive and negative NSIP were not analyzed. No case was diagnosed with PM/DM during the median observation period of 37.8 months (range 17.0–64.6) regardless of the results for anti-synthetase. Numbers of patients who met the criteria of UCTD in anti-synthetase positive and negative groups were 6 of 13 and 21 of 185, respectively. If patients with UCTD were excluded, the prevalence of anti-synthetase was 7 of 171 (4.1%).

Laboratory data, physiological measurements and BAL findings

In the anti-synthetase positive group, erythrocyte sedimentation rate (ESR) was significantly higher, and positive results for rheumatoid factor (RF) and anti–SS–A/Ro were more frequently seen when compared with the anti-synthetase negative group (Table 2). RF and anti-SSA/Ro were each concomitant with anti-synthetase in 4 cases (Table 2). There was no difference in the frequencies of other CTD-specific autoantibodies between ARS-Ab positive and negative groups (data not shown). The partial pressure of arterial oxygen (PaO_2) was significantly higher in the anti-synthetase positive group than in the anti-synthetase negative group ($p = 0.04$). The ratio of CD4+ to CD8+ T lymphocytes in the BAL fluids was predominantly <1 in the anti-synthetase positive group and >1 in the anti-synthetase negative group ($p < 0.01$), though the differences in differential cell counts were not statistically significant.

Pathological classification and findings

Biopsy specimens were obtained from 44 patients in this study; 8 were from anti-synthetase positive patients and 36 were from anti-synthetase negative patients. The numbers of patients who were diagnosed with NSIP and UIP were 11 and 30 respectively. The NSIP pattern was the most predominant (6/8, 75%) in the anti-synthetase positive group. (Table 3). Lymphoid follicles were observed in 7 of 8 cases (87.5%). Fig. 2 illustrates a typical case in the anti-synthetase positive group: observation of a pathological NSIP pattern with lymphoid follicles. Two cases in the anti-synthetase positive group were classified as pathological UIP pattern. While temporal heterogeneity, microscopic

Table 2 Laboratory test results at initial presentation.

Measurements		Anti-synthetase positive (n = 13)		Anti-synthetase negative (n = 185)		p-value
WBC	(/μl)	7300	[5850–12200]	7000	[5700–8600]	0.33
ESR	(mm/hr)	48.0	[33.0–67.0]	22.0	[12.0–41.5]	0.02*
CRP	(mg/dl)	0.9	[0.3–1.6]	0.2	[0.0–0.8]	0.03*
CPK[a]	(IU/L)	67	[39.3–113]	90	[60–121]	0.24
ALD	(IU/L)	5.6	[4.0–8.6]	4.8	[3.7–6.1]	0.27
LDH	(IU/L)	255	[219–342]	233	[198–275]	0.17
ANA(>x40)	(n)	7/12	(58.3%)	74/174	(42.5%)	0.37
RF(>x15)	(n)	4/8	(50.0%)	23/135	(17.0%)	0.04*
Anti–SS–A/Ro (>15.6)	(n)	4/11	(36.4%)	8/129	(6.2%)	<0.01†
Anti–SS–B/La (>10.0)	(n)	1/11	(9.1%)	3/130	(2.3%)	0.28
$PaCO_2$	(mmHg)	41.7	[36.8–44.6]	41.4	[38.4–44.4]	0.70
PaO_2	(mmHg)	85.4	[81.7–90.7]	78.1	[69.6–87.4]	0.04*
% VC	(%)	76.7	[63.5–100.7]	87.9	[71.2–101.5]	0.40
% FVC	(%)	74.9	[62.6–95.9]	87.6	[68.5–99.9]	0.32
% TLC	(%)	72.7	[62.1–90.8]	70.0	[57.3–82.3]	0.54
% RV	(%)	79.6	[71.9–92.5]	67.9	[51.0–87.6]	0.07
% Dlco	(%)	51.9	[35.8–76.0]	49.8	[37.0–62.6]	0.70
BALF findings[b]		(n = 9)		(n = 99)		
Total cell count	(/μl)	210	[123–293]	200	[100–400]	0.62
Neutrophil	(%)	4.0	[0.3–8.0]	6.0	[3.0–14.0]	0.15
Lymphocyte	(%)	20.0	[14.3–51.8]	15.5	[6.7–34.8]	0.14
Macrophage	(%)	61.5	[35.0–79.3]	67.0	[40.5–82.0]	0.75
Eosinophil	(%)	2.0	[1.1–7.3]	3.0	[0.5–5.0]	0.71
CD4/CD8 <1 (n)		7	(77.8)	30	(30.9)	<0.01†

Abbreviations: ALD = aldolase; ANA = antinuclear antibody; anti-synthetase = autoantibody to aminoacyl-tRNA synthetase; BALF = bronchioalveolar lavage fluid; CPK = creatine phosphokinase; CRP = C-reactive protein; Dlco = diffusion capacity; Dlco/VA = diffusion capacity per ESR = erythrocyte sedimentation rate; FVC = forced vital capacity; LDH = lactate dehydrogenase; PaCO2 = partial pressure of arterial carbon dioxide; PaO2 = partial pressure of arterial oxygen; RF = rheumatoid factor; RV = residual volume; TLC = total lung capacity; VA = alveolar ventilation; VC = vital capacity; WBC = white blood cell count. Numbers are expressed as median [25%–75% interquartile] or number of data (percent). Comparison was made by Fisher's exact test; $p < 0.05$ *, $p < 0.01$ †.

[a] Normal range of CPK is 35–141(IU/L).

[b] Number of patients: 9 in anti-synthetase positive and 99 in anti-synthetase negative patients.

honeycombing, and subpleural dense fibrosis suggested a UIP pattern (Fig. 3a), the observed moderate cellular infiltrate was different from a typical UIP (Fig. 3b). Histological UIP with lymphoid follicles were diagnosed in a total of 5 cases, and 2 were positive for the antibody. Therefore, among patients with NSIP or histological UIP with lymphoid follicles, the prevalence of anti-synthetase antibody was 8/16 (50%).

HRCT findings in the anti-synthetase positive group

HRCT findings in anti-synthetase positive cases are shown in Table 4. Interobserver variability (κ coefficient) ranged from 0.60 to 1.0. Abnormal findings were distributed predominantly in lower lung fields. Pleural irregularities and/or prominent interlobular septa, reticulation, ground glass opacity, and traction bronchiectasis or bronchiolectasis were observed in more than 80% of cases. Consolidation, subpleural lines, and irregular peribronchovascular thickening were observed predominantly in lower lung fields in 50% of cases. Honeycombing was not seen in any case. Even in cases with pathological diagnosis of UIP pattern, radiological findings did not follow a typical IPF/UIP pattern (Fig. 3c). Centrilobular nodular opacity was found in a substantial number of anti-synthetase positive cases (Fig. 4).

Discussion

We have demonstrated the prevalence of anti-synthetase among a substantial number of patients with IIPs to be 6.6% (13 of 198 cases). Measurements were carried out regardless of the presence or absence of extrapulmonary ASS-features. The anti-synthetase positive population was younger at onset and had an almost equal sex ratio. Nonspecific interstitial pneumonia (NSIP) pattern was a predominant pathological diagnosis in the anti-synthetase positive group and the most common HRCT findings were diffuse ground glass opacities in all lung fields and traction bronchiectasis in both lower lung fields. Even in cases which were pathologically diagnosed with UIP, radiographic features were not typical of IPF/UIP.

The patients with ILD and positive anti-synthetase are in the category of ASS without myositis. A previous study

Table 3 Summary of clinical manifestations, autoantibody profiles, pathological findings, and fulfillment of criteria of UCTD in patients with positive autoantibodies to aminoacyl-tRNA synthetases (anti-synthetase).

case no.	age and sex	anti-synthetase	CTD signs or symptoms	ANA	RF	other autoantibodies, inflammatory markers	pathological classification and notable findings	UCTD[b]
1	54 F[a]	EJ	monoarthritis	–	+	anti-CCP, SSA	fNSIP, lymphoid follicle	yes
2	43 M	EJ	–	x40	+	–	cNSIP, lymphoid follicle	–
3	61 M	EJ	–	x80	–	–	cNSIP	–
4	60 F	EJ	–	x40	–	–	UIP, cellular infiltrate, lymphoid follicle	–
5	41 M	EJ	finger tip hyperkeratosis	–	–	positive ESR, CRP		yes
6	62 M[a]	EJ	finger tip eczema, articular swelling	x40	–	anti-CCP		yes
7	43 M	PL12	fever, body weight loss, cutaneous manifestation	x40	+	anti-SSA, SSB		yes
8	52 M	PL12	–	x320	+	anti-SSA	fNSIP, lymphoid follicle	–
9	60 F	PL12	–	x40	–	–	UIP, cellular infiltrate, lymphoid follicle	–
10	54 M	Jo-1	finger tip desquamation	–	–	positive CRP		yes
11	58 F	PL7	sicca	–	–	–	fNSIP, lymphoid follicle	–
12	45 F	OJ	Raynaud's phenomenon	–	–	anti-SSA		yes
13	60 F	KS	–	–	–	–	cNSIP, lymphoid follicle	–

Abbreviation: ANA = antinuclear antibody; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; c/f NSIP = cellular/fibrosing nonspecific interstitial pneumonia; RF = rheumatoid factor; UCTD = undifferentiated connective tissue disease; UIP = usual interstitial pneumonia.

[a] These two cases were retrospectively categorized into early arthritis according to the new ACR/EULAR criteria published in 2010. These two cases were not classified into RA according to the 1987 ACR criteria. At the study entry, the new ACR/EULAR diagnostic criteria had not yet been published; therefore we did not exclude these two cases and listed them as anti-synthetase syndrome.

[b] The criteria of UCTD were by Kinder et al published in 2007.

showed that anti-synthetase was detected in a proportion of type 1 diabetes patients[21]; however another report showed its 100% specificity to ASS using the IPP method.[22] In this study, no study subject had concurrent type 1 diabetes. As in previous studies, the prevalence of pulmonary manifestation varied among the different antibodies.[4,23–26] Anti-Jo-1 is the most prevalent in patients whose muscular symptoms are obvious, and anti-PL7 and anti-EJ show the next highest prevalence.[3] However, anti-Jo-1 is less prevalent in patients whose muscular symptoms are absent or obscure.[7,27] Patients with positive anti-PL-12, anti-KS or anti-OJ tend to have ILD predominantly, rather than myositis.[27–30] In this study, anti-EJ was observed most frequently in as much as 2.7 percent of patients, while the prevalence of anti-Jo-1 was small. This result may reflect the difference in overall frequencies of each antibody among anti-synthetase positive patients and suggests that anti-Jo-1 antibody is relatively rare among anti-synthetase positive patients without myositis.

As to the clinical associations, younger age and increased CRP or ESR may be characteristic features of anti-synthetase positive cases. Among extrapulmonary features of ASS, arthralgia, joint deformity, or cutaneous symptoms were significantly observed in anti-synthetase positive cases. Extrapulmonary features of ASS including Raynaud's phenomenon, arthralgia, or muscular symptoms were absent in about half of anti-synthetase positive cases; this demonstrates the difficulty of deciding whether to test for screening of anti-synthetase based on presence of extrapulmonary symptoms of ASS alone. RF or anti-SSA is often concomitant in patients with anti-synthetase positive IPs, as reported in previous studies.[8,30] Furthermore, it is reported that coexistence of anti-SSA/Ro and anti-synthetase, particularly anti-Jo-1, is predictive of a more severe fibrosis score in HRCT and a reduced treatment response to immunosuppressants.[31,32] Therefore, measurement of anti-synthetase merits consideration in anti-SSA/Ro positive cases. Regarding the pulmonary function test results and BAL fluid findings, predicting the presence of anti-synthetase is difficult from these practical measurements. All the anti-synthetase positive cases survived during the study period; however the observation period was shorter in the anti-synthetase positive group. Because of the higher proportion of NSIP in the anti-synthetase positive group, their survival rate was expected to be better than in those without the antibody. However, this study did not show a conclusive difference in survival.

The impact of anti-synthetase on treatment response and prognosis, especially with common lung histopathology, needs to be defined in a future study involving a longer observation period.

The pathological diagnosis in ASS was predominantly NSIP. Even pathologically confirmed UIP showed some atypical features (Fig. 3b). Lymphoid follicles were remarkable findings in the anti-synthetase positive group (Table 3, Fig. 2c). Lymphoid follicle is commonly seen in lung biopsy specimens obtained from a case with CTD, particularly rheumatoid arthritis.[33] A recent study demonstrated that the germinal center score (i.e., number of lymphoid follicles per microscopic field) was the most distinguishing feature between CTD-related interstitial pneumonia (IP) and IPF/UIP.[34] The results of the present study

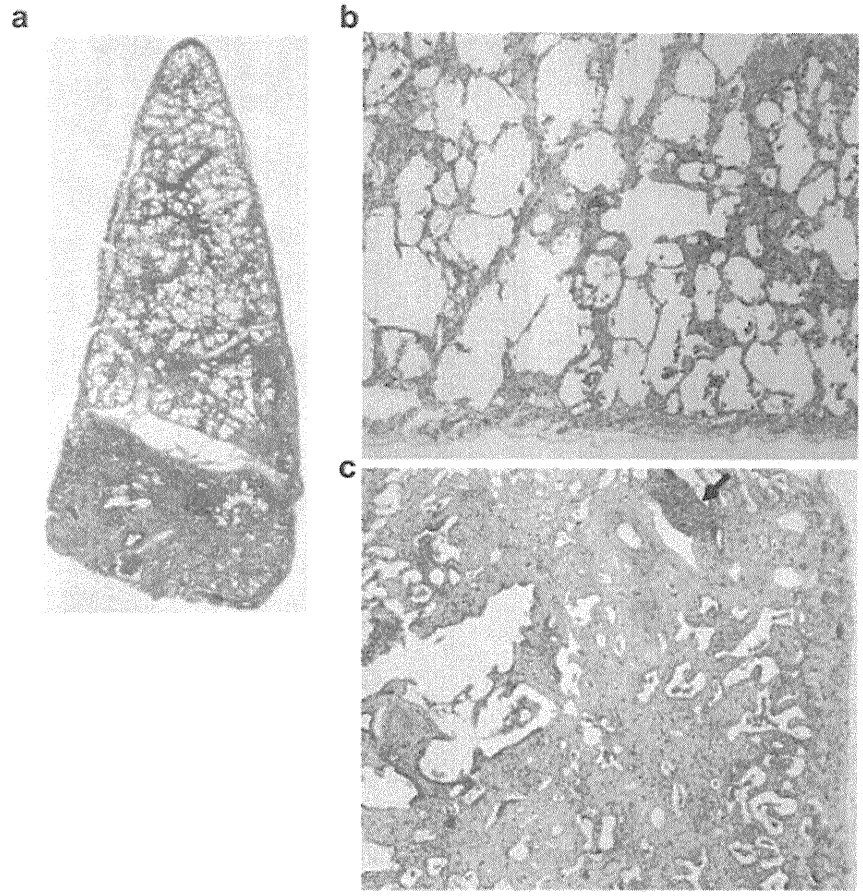

Figure 2 Pathological images in a patient with positive antibody to aminoacyl-tRNA synthetases (anti-synthetase). Pathological classification is nonspecific interstitial pneumonia (NSIP). 2-a) Low magnification view shows varying portions of inflammation and fibrosis distributed uniformly. 2-b) Lung architecture is generally preserved and there is mild to moderate interstitial fibrosis with cellular infiltrate. 2-c) In the more fibrotic area, the fibrosis is relatively loose. A lymphoid follicle and cellular infiltrate are also seen (arrow). No honeycombing is seen.

suggest that lymphoid follicle is characteristic also in anti-synthetase positive cases.

Chest CT revealed ground glass opacity distributed in all lung fields, traction bronchiectasis in lower lung fields and lack of honeycombing, all of which constitute essential HRCT findings characteristic of a non-UIP pattern. The HRCT findings of the current study were consistent with previous reports on interstitial pneumonia associated with PM/DM.[18] Our results were also in agreement with the American Thoracic Society report on NSIP, which found that the most consistent HRCT findings of NSIP are reticular opacities and traction bronchiectasis with lower lung zone predominance.[20] In idiopathic pulmonary fibrosis or idiopathic NSIP, centrilobular nodular opacity is not mentioned[1]; however in CTD-related lung disease such as rheumatoid lung disease, nodules are observed in pathological or radiological UIP or NSIP.[35] 30—40% of such nodules have been described as exhibiting centrilobular distribution. Centrilobular nodular opacity may be a finding characteristic of HRCT in anti-synthetase positive IIPs, but a rigid radio-pathological correlation was not established in this study.

Some cases with IPs exhibit specific autoantibodies or CTD signs or symptoms while they do not meet any specific criteria for CTD. A recent study reported that a large proportion of patients with idiopathic NSIP fulfilled the criteria for UCTD.[17] Indeed, 6 anti-synthetase positive and 21 anti-synthetase negative patients in our series also fulfilled the same criteria for UCTD, even without measurement of anti-synthetase. These cases were included in the study population because some patients with UCTD may be thought to overlap with ASS. Additionally, diagnostic criteria have yet to be fully established.[36,37] Some rheumatologists consider that cases with any disease-specific autoantibodies, including anti-synthetase, should be excluded from UCTD.[36]

Our results suggest that ASS-associated IPs have features in common with CTD-associated IPs but distinct from other IIPs, even if clinical CTD symptoms are subtle. Measurement of anti-synthetase may enable us to pick up such characteristics of otherwise "IIPs" cases, which may comprise a distinct entity. However, a larger cohort study should be conducted to address the best classification of such "IIPs" using certain clinical or serological CTD features.

All the anti-synthetase positive patients in this study presented with non-UIP pattern on HRCT and the prevalence of anti-synthetase antibody was as high as 50% among histological NSIP or UIP with lymphoid follicles. Thus, anti-

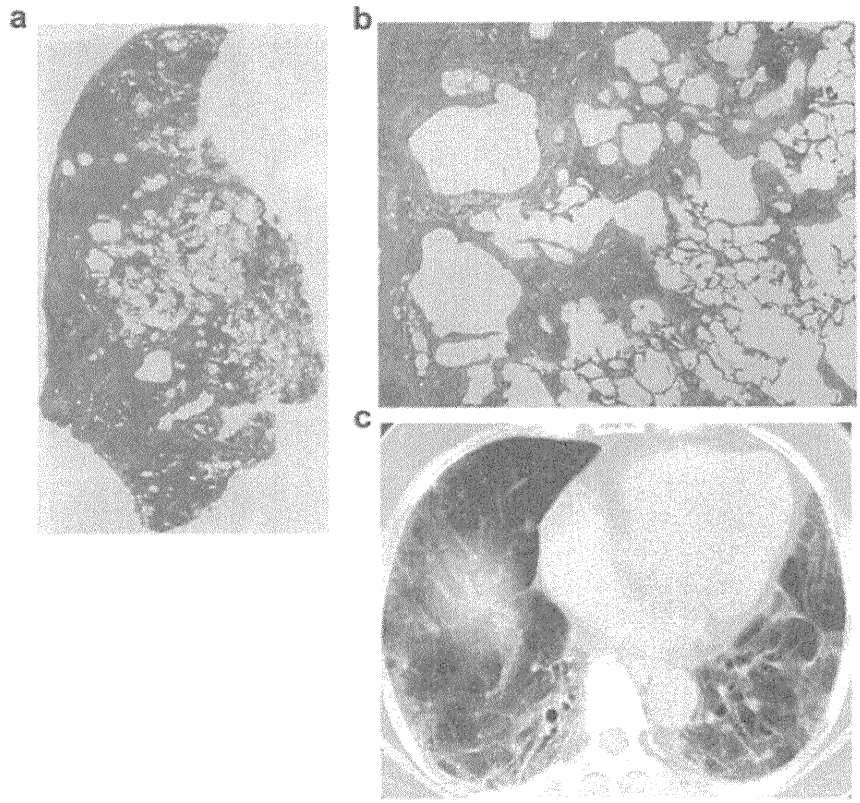

Figure 3 Pathological and radiographic images in a patient with pathological diagnosis of usual interstitial pneumonia (UIP). 3-a) Low magnification view shows subpleural dense fibrosis and normal alveolar architecture distributed in a patchy manner. 3-b) Fibrotic area shows temporal heterogeneity and relatively normal alveolar architecture is adjacent to the dense fibrosis. Fibroblastic foci are scattered. Mild to moderate cellular infiltrate is also seen. 3-c) Radiographic findings of the CT in lower lung fields show ground glass opacities, irregular peribronchovascular thickening and traction bronchiectasis, but do not reveal honeycombing.

synthetase antibodies should be screened for in patients with non-UIP pattern on HRCT, or histological NSIP or UIP with lymphoid follicles. Although all patients with radiologically typical UIP in our series were negative for anti-synthetase antibodies, our results were not conclusive regarding the significance of screening of these antibodies in such patients because of the relatively small sample size. A larger-scale study should be conducted to define the best candidates for anti-synthetase screening.

Previous reports have shown successful treatment of corticosteroid-resistant anti-synthetase positive ILD with immunomodulatory agents such as cyclosporin and

Table 4 Frequency of HRCT findings in patients with positive autoantibodies to aminoacyl-tRNA synthetases (anti-synthetase).

HRCT findings	κ coefficient[a]	Right[b]			Left[b]		
		upper	middle	lower	upper	middle	lower
Pleural irregularities and/or prominent interlobular septa	0.6	11 (85)	10 (77)	11 (85)	9 (69)	11 (85)	10 (77)
Reticulation	0.63	11 (85)	10 (77)	11 (85)	9 (69)	11 (85)	10 (77)
Ground glass opacity	0.67	8 (62)	12 (92)	13 (100)	8 (62)	12 (92)	13 (100)
Consolidation	0.67	0 (0)	2 (15)	5 (38)	0 (0)	1 (8)	7 (54)
Subpleural lines	0.66	3 (23)	6 (46)	8 (62)	3 (23)	6 (46)	9 (69)
Centrilobular nodular opacity	0.62	9 (69)	10 (77)	6 (46)	9 (69)	9 (69)	6 (46)
Irregular peribronchovascular thickening	0.92	0 (0)	1 (8)	6 (46)	0 (0)	1 (8)	7 (54)
Traction bronchiectasis and bronchiolectasis	0.73	1 (8)	6 (46)	11 (85)	0 (0)	6 (46)	10 (77)
Honeycombing	1.0	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] Kappa coefficient of the individual results of two readers.
[b] Data were expressed as number of patients (percent) judged through consensus to have the respective findings.

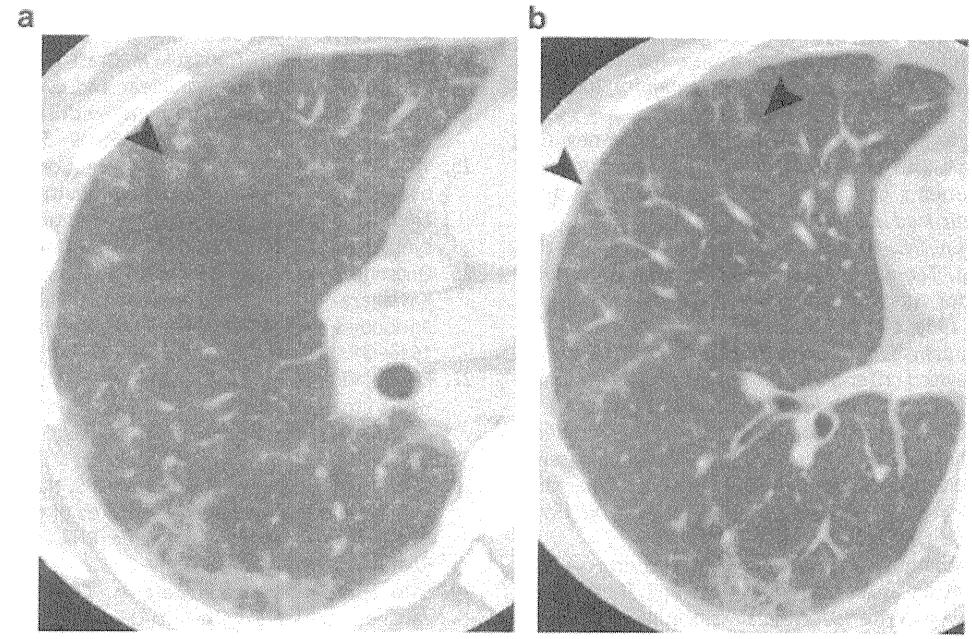

Figure 4 HRCT findings of centrilobular nodular opacity in an anti-synthetase positive case. Small-size nodules with ground glass densities are distributed in centrilobules (arrowhead) in the right middle lung (4-a) and in the right lower lung (4-b), respectively.

tacrolimus.[38] Considering the T lymphocyte involvement in the pathogenesis of ASS,[39] different treatment regimens may be applied for anti-synthetase positive ILD. In addition, all anti-synthetase positive cases have survived in the present study, suggesting that anti-synthetase positive IIPs may have a better prognosis. Although further examinations are necessary to confirm these hypotheses, we should stress the importance of measuring anti-synthetase in IIP for the identification of specific subgroups.

A limitation to our study was the retrospective study design. We could not enroll adequate numbers of patients who had obtained a pathological diagnosis of NSIP, to compare pathological and radiographic features in accordance with the anti-synthetase results. We recruited consecutive cases with IIPs regardless of the presence or absence of lung pathology in a search for the prevalence and characteristics of the anti-synthetase positive subpopulation. A large, prospective, longitudinal cohort of anti-synthetase positive cases would be required to characterize this clinical entity, incorporating treatment choice, response and survival.

In conclusion, an anti-synthetase positive subpopulation was not rare among IIPs. Anti-synthetase should be screened for in IIP patients, particularly in those with pathological NSIP or UIP with lymphoid follicles, even if no suggestive extrapulmonary manifestation exists.

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Conflict of interest

None of the authors have any financial or personal relationships with other people or organizations that could inappropriately influence (bias) the work reflected in this manuscript.

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CASE BASED REVIEW

A case of antisynthetase syndrome in a rheumatoid arthritis patient with anti-PL-12 antibody following treatment with etanercept

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Abstract In our earlier study, we had reported the case of a patient with rheumatoid arthritis (RA), who had anti-Jo-1 antibodies. This patient had received etanercept (ETN) therapy for RA, after which she had developed overt polymyositis (PM). Although various autoimmune phenomena, including lupus-like diseases, vasculitides, or psoriatic skin lesions, are associated with antitumor necrosis factor (TNF) therapy, the development of PM/dermatomyositis (DM) or antisynthetase syndrome following anti-TNF therapy is extremely rare. Here, we report a case of an RA patient with anti-PL-12 antibodies, who received ETN therapy and subsequently developed the antisynthetase syndrome. She recovered when ETN therapy was withdrawn and high-dose corticosteroid was administered. To date, there have been only five reported cases of RA patients with anti-Jo-1 antibodies who developed overt PM/DM following anti-TNF therapy and only one case of antisynthetase syndrome in an RA patient with anti-PL-7 antibodies. Our patients and the abovementioned reports strongly suggest that onset of overt PM/DM or antisynthetase syndrome in RA patients with anti-aminoacyl tRNA synthetase antibodies is associated with anti-TNF therapy.

Keywords Adverse drug effect · Anti-ARS antibodies · Anti-TNF therapy · Polymyositis/dermatomyositis

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Introduction

We had previously reported the case of a patient with rheumatoid arthritis (RA), who had been positive for anti-Jo-1 antibodies and had developed polymyositis (PM) following etanercept (ETN) therapy for RA [1]. Although various autoimmune phenomena, including lupus-like diseases, vasculitides, or psoriatic skin lesions, associated with antitumor necrosis factor (TNF) therapy have been reported [2], the development of PM/dermatomyositis (DM) following anti-TNF therapy is extremely rare. Only five such cases have been reported, including our previous report [1, 3–7], and only one report has shown the association of antisynthetase syndrome with anti-TNF therapy against RA [7]. Here, we report a case of an RA patient with anti-PL-12 antibodies (an anti-aminoacyl tRNA synthetase (ARS) antibody) who was successfully treated with ETN therapy for active RA; however, the patient subsequently developed overt antisynthetase syndrome.

Case report

A 63-year-old woman, who had been treated for nonspecific interstitial pneumonia (NSIP) with prednisolone (PSL) and cyclosporine for 3 years, was diagnosed with RA because she had morning stiffness, systemic joint swelling and tenderness, elevated levels of inflammatory markers such as C-reactive protein (CRP), and elevated erythrocyte sedimentation rate (ESR). Moreover, her serum was positive for rheumatoid factor (RF) and anticitrullinated cyclic peptide (CCP) antibody. At the time of RA diagnosis, NSIP was not active, and she did not have any respiratory symptoms while she was receiving a maintenance dose of PSL without concomitant

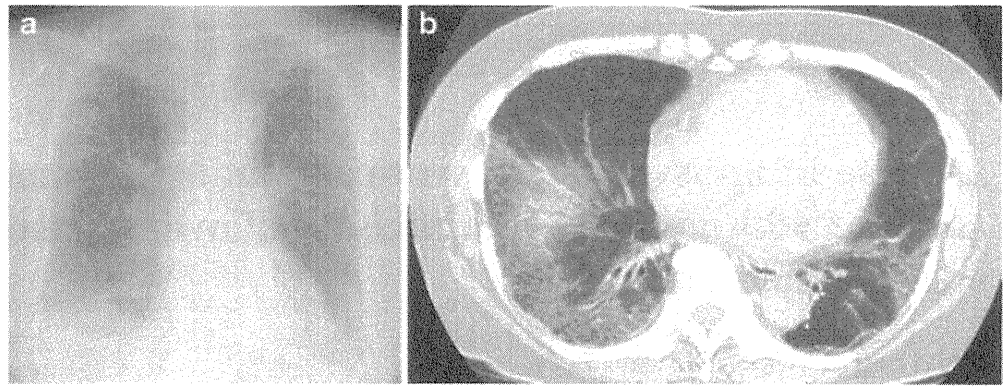

Fig. 1 a Chest X-ray and b computed tomography scan on admission, showing reticulonodular shadows at the base of both the lungs

administration of cyclosporine. Although tacrolimus (TAC) was chosen as the disease-modifying antirheumatic drug (DMARD), it was only minimally effective for the treatment of arthritis, despite adequate duration of treatment. The maintenance dose of PSL was 10 mg daily. ETN (25 mg, twice daily) was administered along with TAC and PSL for the adequate control of active arthritis. Consequently, the joint swelling and tenderness subsided and the levels of the inflammatory markers decreased rapidly. However, systemic arthralgia recurred 2 months after the initiation of ETN therapy; this was accompanied by low-grade fever and erythema on the trunk. In addition, the chest roentgenogram revealed exacerbation of ground-glass opacities. Since adverse drug reactions were suspected, ETN therapy was discontinued, but the symptoms persisted. The patient was admitted for further examination and treatment.

On admission, she was mildly febrile, and we observed a flare-up of systemic joint swelling and tenderness after ETN cessation. She experienced dyspnea on exertion, but the arterial oxygen saturation and the findings of the pulmonary function test were almost normal. Trunk erythema disappeared completely, and eruptions, including Gottron's papule, heliotrope rash, or mechanic's hand, were not detected. She had no muscular symptoms, and manual muscle tests yielded normal findings. Further, nerve conduction study and electromyography did not reveal any specific abnormal findings. Plain radiography of the joints of the hands and feet revealed periarticular osteoporosis, multiple bony erosions, and joint space narrowing, thereby indicating stage II RA of the Steinbrocker classification. Chest roentgenography and computed tomography showed exacerbation of interstitial markings at the base of both the lungs (Fig. 1). Laboratory examinations revealed that the CRP level was 6.9 mg/dL, ESR 85 mm/h, and IgG level 2,538 mg/dL. The creatine kinase level was normal. The autoantibody profiles showed IgM-RF level of 284 IU/mL, anti-CCP antibodies of >100 U/mL, 1:320 fluorescent antinuclear antibodies with speckled and nucleolar patterns, and anti-Sjogren's syndrome antigen A (anti-SS-A/Ro) antibodies of 84.8 U/mL. In addition, the analysis of her serum by the RNA-immunoprecipitation (IPP) method with HeLa cell extracts revealed that her serum was positive for anti-PL-12 antibody. On the basis of these findings, she was diagnosed with antisynthetase syndrome. Treatment with 1 mg/kg of PSL was highly successful (Fig. 2): Dyspnea subsided and radiography images showed rapid disappearance of interstitial markings. Presently, she is healthy and

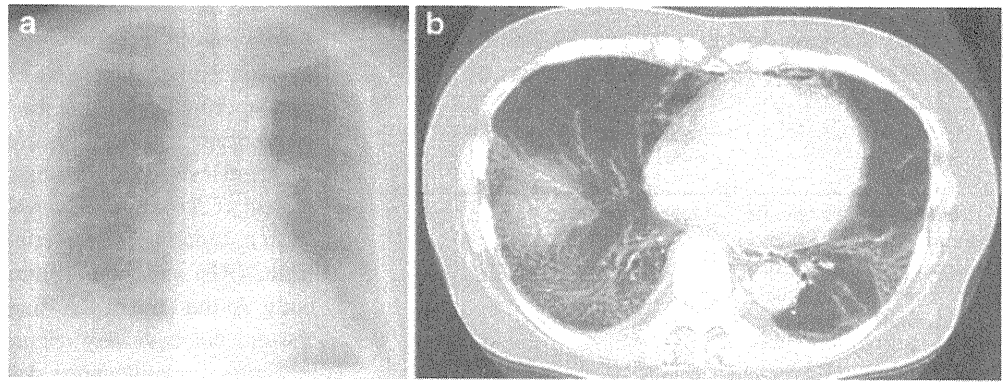

Fig. 2 a Chest X-ray and b computed tomography scan after treatment, showing improvement of interstitial markings

Table 1 Clinical characteristics of RA patients who developed antisynthetase syndrome after anti-TNF Therapy

Authors [referral number]	Harald and Bernald [3]	Musial et al. [4]	Urata et al. [5]	Ishikawa Y et al. [1]	Ishiguro et al. [7]	Present case
Age and sex	44 females	52 females	52 females	58 females	52 males	63 females
RF/CCP	–/–	+/ND	+/ND	+(724.7)/+(>100)	+(1,376)/+(523)	+(64.7)/+(>100)
Disease duration of RA (years)	1	20	33	2	12	0.3
DMARDs	HCQ, MTX	MTX	MTX	BUC, TAC	BUC, MTX,TAC	BUC, TAC
Anti-TNF therapy	Etanercept	Infliximab	Infliximab	Etanercept	Etanercept	Etanercept
Onset from anti-TNF therapy initiation (months)	6	6	9	2	26	2
FANA	1:640, Spe.	1:320, pattern unknown	1:640, Ho./Spe.	1:320, Ho./Nuc.	Negative	1:160, Spe./Nuc.
Antisynthetase antibody	Jo-1	Jo-1	Jo-1	Jo-1	PL-7	PL-12
Fever	ND	Yes	ND	No		Yes
Skin eruptions	Erythematous rash over the extensor surfaces of the MCP, PIP, and DIP joints periungual erythema	ND	ND	No	Heliotrope rash, Gottron's macule	Erythematous rashes on trunk; disappeared without treatment
Exacerbation of ILD	Yes; NSIP	No; UIP	Yes; NSIP	Yes; NSIP	Yes; NSIP	Yes; NSIP
Muscle biopsy	Necrosis, perivascular interstitial infiltration	Diffuse necrosis, inflammatory infiltrates	Size variation, inflammatory infiltrates	Mild inflammatory infiltrates and necrosis	ND	ND
Diagnosis	Dermatomyositis	Polymyositis	Polymyositis	Polymyositis	Antisynthetase syndrome	Antisynthetase syndrome
Treatment[a]	High-dose PSL plus AZP 150 mg and MTX 10 mg/week	MP pulse 1.0 g plus PSL 1 mg/kg	PSL 30 mg plus TAC 3 mg	PSL 1 mg/kg plus MP pulse 0.5 g	PSL 1 mg/kg plus MP pulse 1 g	PSL 1 mg/kg
Treatment outcome	Improvement; NSIP also	Improvement; UIP unchanged	Improvement; NSIP also	Improvement; NSIP also	Improvement; NSIP also	Improvement; NSIP also

ND not done or not described, *DMARDs* disease-modifying antirheumatic drugs, *HCQ* hydroxychloroquine, *MTX* methotrexate, *BUC* bucillamine, *TAC* tacrolimus, *Spe.* speckled, *Ho.* homogenous, *Nuc.* nucleolar, *NSIP* nonspecific interstitial pneumonia, *UIP* usual interstitial pneumonia, *AZP* azathioprine, *MP* methylprednisolon

[a] All treatment include withdrawal of anti-TNF therapy

she is receiving a maintenance dose of PSL, and the disease has not recurred. Although TAC is only a DMARD used for the control of RA, arthritis has never recurred, and no additional treatment agents, including biologic agents, are required.

Discussion

Antisynthetase syndrome is characterized by many clinical features, including mild to moderate grade fever; polyarthritis, usually without joint destruction; interstitial lung disease (ILD), especially NSIP; and characteristic skin eruptions called mechanic's hand, with or without inflammatory myositis in patients with anti-ARS antibodies [8]. Anti-ARS antibodies recognize ARSs in many cells, including myocytes; six major anti-ARS antibodies, of which the antigens are histidyl-(Jo-1) [9], alanyl-(PL-12) [10], threonyl-(PL-7) [11], isoleucyl-(OJ) [12], glycyl-(EJ) [12], and asparaginyl-tRNA synthetase (KS) [13], have been reported to date. However, the precise pathophysiological roles of these antigens have not yet been clarified.

Only five cases of PM/DM associated with anti-TNF therapy for RA have been reported so far [1, 3–6]. Four of these five patients, including ours, had anti-Jo-1 antibody before the initiation of anti-TNF therapy, and PM/DM resolved after the cessation of anti-TNF therapy and the initiation of corticosteroid (CS) therapy. In addition, three of the four patients and our patient had ILD, which exacerbated after anti-TNF therapy and subsided after CS therapy (Table 1). Anti-TNF agents used in these reports included infliximab and ETN. New onsets or flare of ILDs following anti-TNF therapies have also been reported, most of which were RA cases [2]. Although the profiles of anti-ARS antibodies in these RA patients were unclear, it is possible that some of the patients had anti-ARS antibodies that may have been associated with exacerbation of ILD. In addition, a Japanese article recently showed a case of an RA patient with anti-PL-7 antibody: This patient had developed overt antisynthetase syndrome following treatment with ETN for RA, but his condition had improved after he discontinued ETN therapy and began treatment with 1 mg/kg of PSL [7]. This report is very similar to our case in its clinical course. Although present case did not show myositis, patients with anti-Jo-1 antibody show myositis more frequently than those with anti-PL-12 antibody [8]. On the other hand, anti-PL-12 antibody is more associated with ILD than with myositis [14]. Although several common clinical features characterize the antisynthetase syndrome, there are also some differences among patients with different anti-ARS antibodies, which will be clarified in a future study that shows the pathobiological roles of each antibody.

RA patients with anti-ARS antibodies may have a risk for developing antisynthetase syndrome after undergoing anti-TNF therapies. Screening the profiles of anti-ARS antibodies in RA patients is not recommended because RNA-IPP is currently not performed routinely, and the frequency of positivity of the anti-ARS antibodies among RA patients is unclear. However, if anti-TNF therapy is initiated for the treatment of RA, cautions should be taken for patients showing symptoms of the antisynthetase syndrome.

Disclosures None

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RESEARCH ARTICLE Open Access

Epidemiologic study of clinically amyopathic dermatomyositis and anti-melanoma differentiation-associated gene 5 antibodies in central Japan

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Abstract

Introduction: Several reports have found the onset or activity of inflammatory myopathies to show spatial clustering and seasonal association. We recently detected autoantibodies against melanoma differentiation-associated gene 5 (MDA-5) in more than 20% of patients with dermatomyositis. Anti-MDA-5 antibodies were associated with the presence of rapidly progressive interstitial lung disease in clinically amyopathic dermatomyositis (CADM). The present study aims to assess the growing prevalence of CADM and the geographical incidence of anti-MDA-5-positive patients.

Methods: We reviewed medical charts and examined the presence of anti-MDA-5 antibodies in 95 patients, including 36 CADM patients. Sera were obtained from 1994 through 2011. Statistical analyses were performed to assess whether CADM development and the presence of anti-MDA-5 antibodies were associated with various parameters, including age at disease onset, season of onset, annual positivity, and population of resident city.

Results: Tertiles based on the year when the sera were collected showed increasing tendencies of CADM and anti-MDA-5-positive patients among all of the dermatomyositis patients. From 1994 to 2010, the relative prevalence of CADM and anti-MDA-5 antibody-positive patients significantly increased. Interestingly, the presence of anti-MDA-5 antibodies in 26 patients was inversely associated with the population of their city of residence.

Conclusions: This is the first study to examine the distribution of anti-MDA-5-positive dermatomyositis phenotypes in Japan. Regional differences in the incidences of these phenotypes would suggest that environmental factors contribute to the production of antibodies against MDA-5, which triggers innate antiviral responses.

Introduction

Idiopathic inflammatory myopathies are a heterogeneous group of autoimmune disorders that target the skeletal muscle and skin. Disease-related death is generally associated with malignancy and interstitial lung disease. The most frequent forms, polymyositis and dermatomyositis (DM), are thought to result from environmental exposure that leads to immune activation in genetically susceptible individuals. Several reports have found the onset or activity of inflammatory myopathies to show spatial clustering and seasonal association [1-5].

A subgroup of DM patients who have typical skin manifestations of DM but little evidence of myositis has been recognized as clinically amyopathic dermatomyositis (CADM) [6]. Although it is still undetermined whether CADM is a distinct clinical entity or just an early phase of classic DM, rapidly progressive interstitial lung disease (ILD) can occur in CADM patients, especially in East Asia [7]. This patient subset with CADM and rapidly progressive ILD has been shown to have specific autoantibodies, originally called anti-CADM-140 antibodies [8]. The target autoantigen is melanoma differentiation-associated gene 5 (MDA-5) [9-11], which

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plays important roles in the innate immune system during RNA virus infections [12].

To better understand this subset of patients, it is important to examine the epidemiologic characteristics of CADM patients with anti-MDA-5 antibodies, whose outcome is often fatal. According to our clinical experiences, we have recently noticed that the prevalence of CADM patients with anti-MDA-5 antibodies seems to be growing, particularly in rural areas. We therefore examined the epidemiologic features of CADM and anti-MDA-5 antibodies in a single cohort of DM patients.

Materials and methods

Patients

We reviewed medical charts and examined the presence of anti-MDA-5 antibodies in 95 Japanese patients (one of them a half-Japanese, half-Filipino boy) with DM, including 36 patients with CADM, 15 patients with cancer-associated DM and 44 patients with classical DM, who were seen by or consulted the Department of Dermatology at Nagoya University Graduate School of Medicine from 1994 to 2011. These patients were diagnosed with DM or CADM based on the criteria of Bohan *et al.* [13] or Sontheimer [6], respectively. In general, CADM presents as typical skin lesions and amyopathy or hypomyopathy that lasts for more than 6 months. The CADM group included patients who developed fatal ILD within the first 6 months after disease onset. Since juvenile DM with rapidly progressive ILD and/or anti-MDA-5 antibodies has been reported in Japan [7,11,14], patients who manifested the disease at < 18 years of age were also included. Patients who were originally seen at other hospitals far outside our area and who then transferred to our hospital were excluded from the present study. Serum samples were obtained from all of the patients between 1 October 1994, the date when we began to build a serum bank of autoimmune rheumatic disease patients, and 30 June 2011. The population data on city of residence in 2010 were obtained from web data published by public offices in 25 cities, eight counties and one village.

The present study was approved by the Ethics Committee of Nagoya University Graduate School of Medicine. This study meets and is in compliance with all ethical standards in medicine. Informed consent including that for publication of the study was obtained from all patients according to the Declaration of Helsinki.

Immunoprecipitation

Anti-MDA-5 antibodies were screened by an immunoprecipitation assay using biotinylated recombinant MDA-5 produced from full-length MDA-5 cDNA using the TnT® T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA) and the Transcend™ Colorimetric Non-Radioactive Translation Detection System (Promega), according to our published protocol [11]. This method was confirmed to produce consistent results based on a standard immunoprecipitation assay using ^{35}S-methionine-labeled cell extracts [11]. Serum samples from 82 patients were already characterized in our previous report [11]. All serum samples were stored at -70°C until the experiments.

Statistical analysis

The subjects were divided into tertiles based on year the sera were collected, age at collection, age at onset, or population of the city of residence, separately, to examine the associations between each of these factors and the development of CADM and the presence of anti-MDA-5 antibodies. The differences and linear trends across the tertiles were assessed using the chi-square test and the Cochran-Armitage trend test, respectively. SPSS version 17.0 for Windows (SPSS Japan Inc., Tokyo, Japan) was used to perform the statistical analysis. $P < 0.05$ was considered significant.

Results

Patient population

Between 1 October 1994 and 30 June 2011, sera from 95 patients with DM were collected. During 1994 sera were drawn from 24 patients, two-thirds of whom had been diagnosed with DM and treated by our department. The mean age at onset was 46.9 years (range: 1 to 80 years) and that at the time of sera collection was 50.2 years (range: 3 to 84 years). There were 67 (70.5%) female patients. Ten patients developed the disease under 18 years of age.

A review of the medical records indicated that 36 patients (28/36, 77.8% female; 5/36, 13.9% juvenile) had CADM. For these 36 patients, the mean age at onset was 44.9 years (range: 1 to 73 years) and that at the time of sera collection was 48.2 years (range: 3 to 84 years). Based on the immunoprecipitation assays, 26 patients (21/26, 80.8% female; 1/26, 3.8% juvenile) had anti-MDA-5 antibodies. For these 26 patients, the mean age at onset was 46.8 years (range: 11 to 66 years) and that at the time of sera collection was 48.2 years (range: 11 to 71 years). Twenty-five patients with anti-MDA-5 antibodies were diagnosed as CADM, and the remaining patient met the criteria for classical DM. All but one of our patients with anti-MDA-5 antibodies had ILD.

To grasp the overall trend, tertile analysis was conducted based on the number of cases for all patients with DM as well as for patients with CADM and those with anti-MDA-5 antibodies (Table 1). The mean ages at onset and at the time of sera collection did not significantly differ among the tertiles (data not shown), but

Table 1 Patient characteristics based on the presence of CADM or anti-MDA-5 antibodies

			CADM			α-MDA-5-positive		
Years of sera collection	Total number of DM patients (M:F)	Mean age at onset (range)	Number (%) of patients (M:F)	*P* value*	Mean age at onset (range)	Number (%) of patients (M:F)	*P* value**	Mean age at onset (range)
T1 (1994 to 1995)	32 (12:20)	47.5 (4 to 80)	6 (18.8%) (2:4)	*P* for difference = 0.012	45.5 (4 to 73)	2 (6.3%) (1:1%)	*P* for difference = 0.003	53 (43 to 63)
T2 (1996 to 2003)	30 (6:24)	50.1 (15 to 79)	12 (40.0%) (1:11)	*P* for trend = 0.003	50.7 (20 to 73)	10 (33.3%) (0:10)	*P* for trend = 0.001	48.9 (20 to 66)
T3 (2004 to 2011)	33 (10:23)	43.6 (1 to 73)	18 (54.5%) (5:13)		40.8 (1 to 69)	14 (42.4%) (4:10)		44.4 (11 to 58)

CADM, clinically amyopathic dermatomyositis; DM, dermatomyositis; M:F, male:female; MDA-5, melanoma differentiation-associated gene 5. *Prevalence of CADM in total DM. **Prevalence of anti-MDA-5 in total DM.

the proportions of CADM and anti-MDA-5-positive patients significantly increased from the first to the third periods of the study.

Annual prevalence of CADM and anti-MDA-5 antibodies

Since many of the patients whose sera were drawn in 1994 had been treated at our hospital, the above tertile analysis was partially biased. Before the sampling in 1994 there may have been some fatal cases of rapidly progressive ILD. Moreover, some CADM patients stopped seeing their doctors due to minor illness. In light of these possibilities, we analyzed only the 72 patients who manifested the disease after 1994, in order to investigate the growing trend of CADM and anti-MDA-5-positive patients (Figure 1). The relative prevalence of both CADM and anti-MDA-5-positive patients among all DM patients was found to have significantly increased (P = 0.029 and P = 0.044, respectively).

Geographical incidence of dermatomyositis patients with anti-MDA-5 antibodies

Our university hospital is in Nagoya (population 2.2 million), the biggest city in central Japan. To clarify the regional differences in a subgroup of patients, we compared the prevalence of CADM and anti-MDA-5-positive patients by tertiles based on the population of the patient's city of residence (Table 2), and we plotted the anti-MDA-5-positive patients on a map (Figure 2). Interestingly, CADM patients were less prevalent in

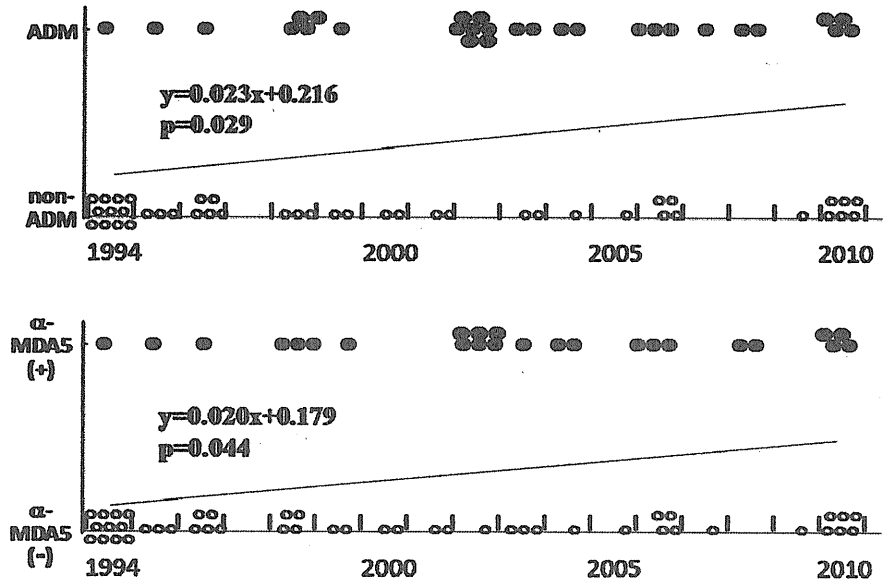

Figure 1 Annual prevalence of patients with clinically amyopathic dermatomyositis or anti-melanoma differentiation-associated gene 5 antibodies. The regression equation is shown, in which the year of disease onset is defined as 1994 = 1, 1995 = 2,..., 2010 = 17 on the *x* axis and the presence or absence of clinically amyopathic dermatomyositis (CADM) or anti-melanoma differentiation-associated gene 5 (anti-MDA-5) antibodies is defined as 1 and 0, respectively, on the *y* axis (*P* for linear trend).

Table 2 Population of the area of residence and the presence of CADM or anti-MDA-5 antibodies

			CADM		α-MDA-5-positive			
Population of area of residence (×1,000)	Total number of DM patients (M:F)	Mean age at onset (range)	Number (%) of patients (M:F)	*P* value*	Mean age at onset (range)	Number (%) of patients (M:F)	*P* value**	Mean age at onset (range)
T1 (0.5 to 108)	31 (4:27)	49.0 (4 to 70)	16 (51.6%) (1:15)	*P* for difference = 0.096	47.3 (4 to 69)	14 (45.2%) (1:13)	*P* for difference = 0.012	48.8 (20 to 66)
T2 (130 to 826)	26 (7:19)	44.0 (9 to 80)	10 (38.5%) (3:7)	*P* for trend = 0.031	40.0 (9 to 59)	7 (26.9%) (3:4)	*P* for trend = 0.003	39.9 (11 to 59)
T3 (2,200)	38 (17:21)	47.3 (1 to 79)	10 (26.3%) (4:6)		45.9 (1 to 73)	5 (13.2%) (1:4)		51.0 (39 to 63)

CADM, clinically amyopathic dermatomyositis; DM, dermatomyositis; M:F, male:female; MDA-5, melanoma differentiation-associated gene 5. *Prevalence of CADM in total DM. **Prevalence of anti-MDA-5 in total DM.

urban areas, but this association was only marginally significant, whereas there were significantly more anti-MDA-5-positive patients in rural areas than in urban ones. Areas northeast and far northwest of Nagoya contained particularly high numbers of patients with anti-MDA-5 antibodies: 10 patients in the northeast, and five patients in the northwest (Figure 2, circular dotted area). These areas had nine and six CADM patients in the northwest and northeast, respectively. All 15 patients with anti-MDA-5 antibodies were natives of the

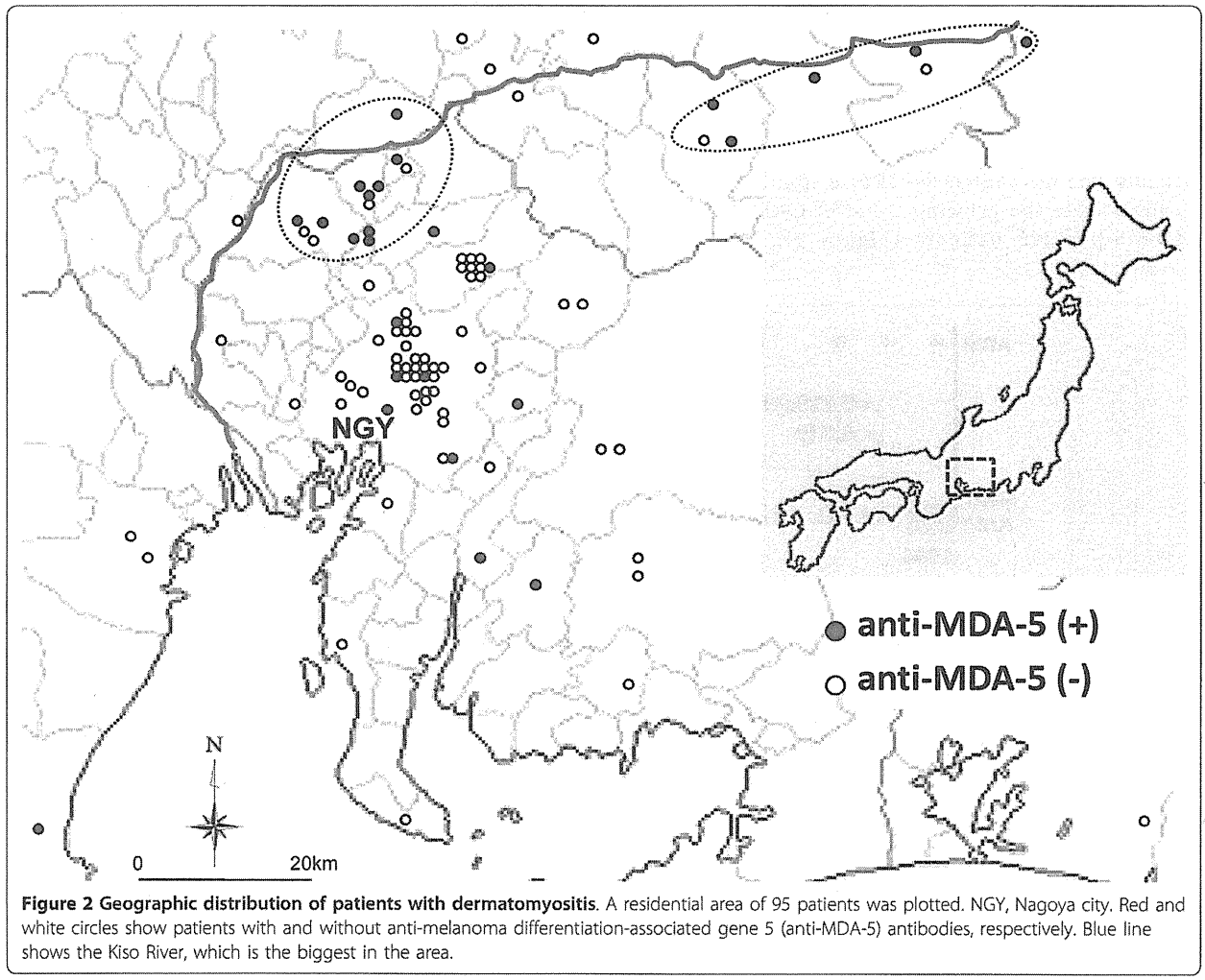

Figure 2 Geographic distribution of patients with dermatomyositis. A residential area of 95 patients was plotted. NGY, Nagoya city. Red and white circles show patients with and without anti-melanoma differentiation-associated gene 5 (anti-MDA-5) antibodies, respectively. Blue line shows the Kiso River, which is the biggest in the area.

area; five and four of these 15 patients had manifested the disease in 2002 and 2010, respectively. Notably, five of the six patients with anti-MDA-5 antibodies whose disease began in 2002 and all four of the patients with anti-MDA-5 antibodies whose disease began in 2010 were from these two areas (Figure 1).

Seasonal onset

The information on seasonality of disease onset was available for 78 patients, including 33 CADM and 25 anti-MDA-5-positive patients. There were no significant seasonal patterns of disease onset in the overall patient group or in the subgroups of male, female, CADM or anti-MDA-5-positive patients (data not shown). However, the incidence of anti-MDA-5 antibodies in areas with populations under 108×10^3, but not in areas with populations over 130×10^3, was the highest in autumn (onset in autumn in areas with populations under 108×10^3 vs. onset in autumn in areas with populations over 130×10^3 = 8/14 vs. 1/11, $P = 0.033$).

Discussion

A Japanese multicenter study confirmed recently that patients with anti-MDA-5 antibodies frequently have CADM with rapidly progressive ILD and a poor prognosis [15]. With increasing awareness of the CADM disease subtype, which was proposed by Sontheimer in the 1990s, we felt not only that the prevalence of CADM is increasing but also that more CADM patients with anti-MDA-5 antibodies have recently been coming from rural areas than from urban ones. To examine these matters statistically, we investigated the prevalence of CADM and anti-MDA-5 antibodies among all of the DM patients.

Because the present study was neither population based nor community based, it is difficult to say that the incidence of CADM is increasing. However, the frequency of anti-MDA-5 antibodies among all DM patients is increasing. Although this autoantibody was only recently characterized [8,9], our initial study found that the serum collected from one patient in 1994 was anti-MDA-5 antibody-positive. Contrary to the increasing prevalence of anti-MDA-5 antibodies, other types of autoantibodies appear to be decreasing. We also characterized the prevalence of anti-transcriptional intermediary factor-1γ antibodies among all patients examined in this study. These antibodies, however, which were detected in 12 patients, showed no significant epidemiological characteristics under the same analysis (data not shown). In our previous study using traditional immunoprecipitation experiments, we did not detect significant decreases in the prevalence of any specific autoantibodies [11]. There is little possibility that the long storage of the sera caused the autoantibodies to become less active, however, because various kinds of DM/polymyositis-specific autoantibodies were found in many of the sera that were drawn in 1994 and 1995 (two patients with anti-transcriptional intermediary factor 1γ, two patients with anti-MJ, two patients with anti-PL-7, one patient with anti-Jo-1, one patient with anti-EJ and one patient with anti-KS; our unpublished observations), along with the anti-MDA-5 antibodies found in the two other patients during this period.

MDA-5 detects some viruses, including picornaviruses, and is involved in the host defense response to infection. Antibodies to coxsackievirus B, a picornavirus, were previously reported to be prevalent in patients with juvenile DM [16]. Although we could not find an epidemiologic study on the environmental levels of picornavirus in our district, the seasonal distribution of viruses in the river water in Nara Prefecture, which is also in central Japan, has been examined [17]. The coxsackievirus B levels peaked there in the summer, and the virus continued to be detected in the autumn and winter. Interestingly, there was a marked increase in the prevalence of anti-MDA-5 antibodies in our study in areas northeast and northwest of Nagoya (Figure 2). These regions are on the Kiso River, which is the biggest river in our area (blue line in Figure 2). In these areas, there was also an accumulation of CADM. It is unlikely that sun exposure strongly contributed to the pathogenesis, because the 15 patients with CADM included only one outdoor worker.

The present study has several limitations because of the small number of study subjects. The time lag between the initial presentation of disease and the clinical assessment should be considered. The interval between disease onset and the time of sera collection in this study was not significantly different, however, between patients with and without CADM, between patients with and without anti-MDA-5 antibodies, or among the tertiles depicted in Table 1 (data not shown), suggesting that the patient follow-up periods did not differ by disease subtype. Since people in rural areas generally have reduced access to specialists, patients with severe illness, such as anti-MDA-5-positive patients, might be more prevalent in rural areas than in urban areas. Moreover, medical practices at a university hospital have an inherent referral bias.

Many reports have suggested that environmental factors play a role in the development of DM and the production of myositis-related autoantibodies (reviewed in [18]). No single factor, however, can explain that development and that production, and the possible growing prevalence of CADM and anti-MDA-5-positive patients. It seems difficult to identify environmental factors that possibly increase the annual prevalence of CADM and anti-MDA-5-positive patients, because patients could have several environmental exposures that have possible interrelationships with genetic risk factors. Various environmental

exposures require confirmation in case-controlled studies to determine which are associated with disease onset and whether these play any role in etiology.

To our knowledge, this is the first epidemiologic study on anti-MDA-5 antibodies. Although it is difficult to draw strong conclusion from a single cohort study, epidemiologic studies play an important role in disease assessment. These studies determine the extent of disease and the natural history within a community, identify potential etiologic factors and enhance our understanding of disease pathogenesis.

Conclusions

Clinically amyopathic dermatomyositis might be growing in prevalence with the increase of anti-MDA-5 antibody-positive patients in central Japan. Regional differences in the incidences of the anti-MDA-5 antibody would suggest that environmental factors contribute to the production of autoantibodies against MDA-5. It will be important to conduct larger population-based studies through multicenter collaboration using DM-specific autoantibodies to define patient groups and clarify the disease etiology associated with environmental factors.

Abbreviations

CADM: clinically amyopathic dermatomyositis; DM: dermatomyositis; ILD: interstitial lung disease; MDA-5: melanoma differentiation-associated gene 5.

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Authors' contributions

YM, KS and KH organized the patient registry. YM and KH performed laboratory assays. KT participated in the design of the study and performed the statistical analysis. YM conceived of the study design and wrote the manuscript with input and consensus from all authors. KS and MA participated in the coordination of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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RESEARCH PAPER

Sera from neuromyelitis optica patients disrupt the blood–brain barrier

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ABSTRACT

Objective In neuromyelitis optica (NMO), the destruction of the blood–brain barrier (BBB) has been considered to be the first step of the disease process. It is unclear whether sera from patients with NMO can open the BBB, and which component of patient sera is most important for this disruption.

Methods The effects of sera from antiaquaporin4 (AQP4) antibody positive NMO patients, multiple sclerosis patients and control subjects were evaluated for expression of tight junction proteins and for transendothelial electrical resistance (TEER) of human brain microvascular endothelial cells (BMECs). Whether antibodies against human BMECs as well as anti-AQP4 antibodies exist in NMO sera was also examined using western blot analysis.

Results Expression of tight junction proteins and TEER in BMECs was significantly decreased after exposure to NMO sera. However, this effect was reversed after application of an antivascular endothelial growth factor (VEGF) neutralising antibody. Antibodies against BMECs other than anti-AQP4 antibodies were found in the sera of NMO patients whereas no specific bands were detected in the sera of healthy and neurological controls. These antibodies apparently disrupt the BBB by increasing the autocrine secretion of VEGF by BMECs themselves. Absorption of the anti-AQP4 antibody by AQP4 transfected astrocytes reduced AQP4 antibody titres but was not associated with a reduction in BBB disruption.

Conclusions Sera from NMO patients reduce expression of tight junction proteins and disrupt the BBB. Autoantibodies against BMECs other than anti-AQP4 antibodies may disrupt the BBB through upregulation of VEGF in BMECs.

INTRODUCTION

Neuromyelitis optica (NMO) is defined as an inflammatory CNS disease predominantly affecting the spinal cord and the optic nerves.[1] This disorder was long regarded as a variant of multiple sclerosis (MS), with distinctive pathological features.[2] A breakthrough in our understanding of NMO was identification of an autoantibody response with high sensitivity and specificity for the disease, which was found to be directed against the astrocytic water channel aquaporin 4 (AQP4).[3] Several studies have suggested that the anti-AQP4 antibody is pathogenic and it also plays a key role in the development of NMO.[4–11]

Circulating anti-AQP4 antibodies need to pass through the blood–brain barrier (BBB) in order to reach the CNS parenchyma, which is the site affected by inflammation in this disease. Initiation of disease by transfer of these antibodies into normal animals has not been achieved to date[12] because the BBB restricts the entry of circulating anti-AQP4 antibodies into the CNS under normal conditions. Although destruction of the BBB causing leakage of anti-AQP4 antibodies and cytokines into the CNS space has been considered as a key step in the development of NMO, it remains unclear which components of patient sera is most important for disruption of the BBB. It is also unclear whether sera from an NMO patient containing circulating anti-AQP4 antibodies can open the BBB because no direct evidence has been presented indicating that the brain microvascular endothelial cells (BMECs), which comprise the BBB, express the AQP4 protein.[13 14] Various circulating inflammatory cytokines, including tumour necrosis factor α (TNFα) and vascular endothelial growth factor (VEGF), which have already been reported to induce disruption of the BBB, may be the candidate molecules leading to the breakdown of the BBB.[15 16] The existence of unknown pathogenic antibodies, apart from anti-AQP4 antibodies, may also cause BBB impairment.

The aim of the current study was to demonstrate the effects of sera from patients with NMO on impairment of BBB function and to clarify the roles of humoral factors, especially antibodies, against human BMECs, in the destruction of the BBB.

MATERIALS AND METHODS

Sera and antibody

The acute phase sera from 14 consecutive NMO patients hospitalised at our institution were studied. All patients met the clinical criteria for NMO spectrum disorders.[17 18] None of the NMO patients had antinuclear antibodies or SS-A/SS-B antibodies. The human anti-AQP4 antibody was detected in all patients by a procedure previously described by Takahashi.[9] Blood samples were obtained within 7 days of onset and stored at −80°C until use. The sera from two patients who began plasma exchange (PE) treatment were also obtained. The acute phase sera from seven patients with conventional MS (C-MS), diagnosed by the McDonald criteria,[19] were also used in this study. The sera from 15 patients with autoimmune inflammatory neurological diseases, including three patients with neuropsychiatric systemic lupus erythematosus (NP-SLE), four patients with dermatomyositis, three patients with myasthenia gravis, three patients with multifocal motor neuropathy and two patients with microscopic polyangiitis were studied as inflammatory

disease controls. All NP-SLE, dermatomyositis and microscopic polyangiitis patients had antinuclear antibodies. In contrast, none of the myasthenia gravis and multifocal motor neuropathy patients had these antibodies. Sera from 12 patients with non-inflammatory neurological diseases, including four patients with amyotrophic lateral sclerosis, two patients with Parkinson's disease, four patients with cervical spondylosis and two patients with multiple system atrophy, were used as neurological disease controls. The sera from 12 healthy individuals also served as normal controls. All sera were incubated at 65°C for 30 min just prior to use. There were no statistically significant differences in the concentrations of IgG between the serum samples of the 14 NMO, 7 MS and 12 normal controls (means±SEM, NMO 1035±517 mg/dl; MS 1090±151 mg/dl; normal controls 1042±225 mg/dl) when the concentration of IgG in each of the samples was measured. The use of the patient's sera for this study was approved by the ethics committee of Yamaguchi University following the principles of the Declaration of Helsinki.

Cell culture and treatment

The immortalised human brain microvascular endothelial cells (BMECs) were generated previously.[20] Briefly, we previously established conditionally immortalised BBB derived endothelial cells, called TY08 cells, harbouring the temperature sensitive SV40 large T antigen (tsA58) protein.[20] The gene product of tsA58 is in an active conformation and binds to p53 at 33°C, thus facilitating the immortalisation of the cells, whereas the conformation of the gene product changes, leading to its degradation and the release of p53 when the cells are grown at 37°C. Therefore, these cells are conditionally immortal. The cells expressed all key tight junctional proteins, such as occludin, claudin-5, ZO-1 and ZO-2, and had low permeabiltity to inulin across monolayers. All of the analyses were determined 3 days after the temperature shift from 33°C to 37°C. Human umblilical vein endothelial cells (HUVECs), human fibroblasts and 293T cells were obtained from the Japan Health Sciences Foundation (Osaka, Japan) and human astrocytes were purchased from Lonza (Walkersville, Maryland, USA). BMECs were treated with culture medium containing 10% patient or healthy control sera in a humidified atmosphere of 5% CO_2/air. BMECs treated with culture medium with 10% fetal bovine serum (Sigma, St. Louis, Missouri, USA) were used as controls. The mRNAs were extracted 24 h later, and total proteins were obtained a day later.

Reagents

The culture medium for BMECs consisted of Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 100 U/ml penicillin (Sigma), 100 μg/ml streptomycin (Sigma), 25 ng/ml amphotericin B (Invitrogen, Grand Island, New York, USA) and 10% fetal bovine serum (Sigma). Polyclonal anticlaudin-5 and antioccludin antibodies were purchased from Zymed (San Francisco, California, USA). The polyclonal antiactin antibody was obtained from Santa Cruz (Santa Cruz, California, USA). The polyclonal anti-transforming growth factor β (TGFβ), anti-VEGF, anti-interleukin (IL)-6, anti-IL-17, anti-interferon γ (IFNγ) and anti-TNFα antibodies were purchased from R&D systems (Minneapolis, Minnesota, USA). Lysates of human claudin-5 transfected 293T cells and control 293T cells were purchased from Santa Cruz. A total of 5 μg of protein lysates were loaded for the western blot analysis.

Quantitative real time PCR analysis

Total RNA was extracted from BMECs using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Single stranded cDNA was created from 40 ng of total RNA using the StrataScript First Strand Synthesis System (Stratagene, Cedar Greek, Texas, USA.). The sequence of each human primer pair and its reference are as follows: sense primer 5′-CTG TTT CCA TAG GCA GAG CG-3′ and antisense primer 5′-AAG CAG ATT CTT AGC CTT CC-3′ for claudin-5[21]; sense primer 5′-TGG GAG TGA ACC CAA CTG CT-3′ and antisense primer 5′-CTT CAG GAA CCG GCG TGG AT-3′ for occludin[22]; and sense primer 5′-GTC AAC GGA TTT GGT CTG TAT T-3′ and antisense primer 5′-AGT CTT CTG GGT GGC AGT GAT-3′ for glyceraldehyde-3-phosphate dehydrogenase.[23] Quantitative real time PCR analyses were performed using a Stratagene Mx3005P (Stratagene) with Full-Velocity SYBR Green QPCR master mix (Stratagene). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal standard. The samples were subjected to PCR analysis using the following cycling parameters: 10 min at 95°C followed by 40 cycles for 15 s at 95°C, 1 min at 60°C and 1 min at 72°C. The standard reaction curve was analysed by the MxPro (Stratagene) software programme and the relative quantity according to standard reaction curve (R_v) was calculated by computer according to the formula $R_v=R_{Gene}/R_{GAPDH}$.

Western blot analysis

Protein samples (10–20 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Biorad) and transferred to nitrocellulose membranes (Amersham, Chalfont, UK). Expression of β-actin was used as an internal standard. The membranes were treated with blocking buffer (5% skimmed milk in 25 mM Tris-HCl, pH 7.6,125 nM NaCl, 0.5% Tween 20) for 1 h at room temperature and incubated with the relevant primary antibodies (dilution 1:100) for 2 h at room temperature. The membranes were then exposed to a peroxidase conjugated secondary antibody (1:2000), followed by a chemiluminescence reagent (Amersham), and exposure to X-Omat S films (Amersham) and quantification of the band intensity was obtained using the Fuji image analysis software package.

Immunocytochemistry

Cultured cells were fixed with 4% paraformaldehyde (Wako, Osaka, Japan) and permeabilised with 100% methanol. Cells were subsequently incubated overnight with 5% serum (as relevant primary antibodies (dilution 1:20)) and then were incubated with a FITC labelled secondary antibody at a dilution of 1:200 for staining. Fluorescence was observed by a fluorescence microscope (Olympus, Tokyo, Japan). The nuclei were stained with DAPI, and the fluorescence was detected with a fluorescence microscope (Olympus). Image stacks were analysed with the localisation module of the Olympus software program (Olympus).

Transendothelial electrical resistance studies

Transwell inserts (pore size 0.4 μm, effective growth area 0.3 cm^2, BD Bioscience, Sparks, Maryland, USA) were coated with rat tail collagen type I (BD Bioscience). Transendothelial electrical resistance (TEER) values of cell layers were measured with a Millicell electrical resistance apparatus (Endohm-6 and EVOM; World Precision Instruments, Sarasota, Florida, USA). BMECs were seeded (1×10^6 cells/insert) on the upper compartment and incubated with each medium (non-conditioned medium used as a control, conditioned medium contained 10% patient sera) for 24 h.

Studies with patient sera preincubated with neutralising antibodies against TNFα, IFNγ, VEGF, TGFβ, IL-6 or IL-17

BMECs were incubated with the sera from eight NMO patients containing 2.0 μg/ml of a neutralising antibody against TNFα,

IFNγ, VEGF, TGFβ, IL-6 or IL-17, or normal rabbit IgG. Total RNA was extracted and the TEER value was measured 24 h later. Total proteins were obtained the next day.

Sera from NMO patients were pretreated with 2.0 μg/ml of a neutralising antibody against TNFα, IFNγ, VEGF, TGFβ, IL-6 or IL-17, or normal rabbit IgG (control antibody) for 6 h at 4°C.

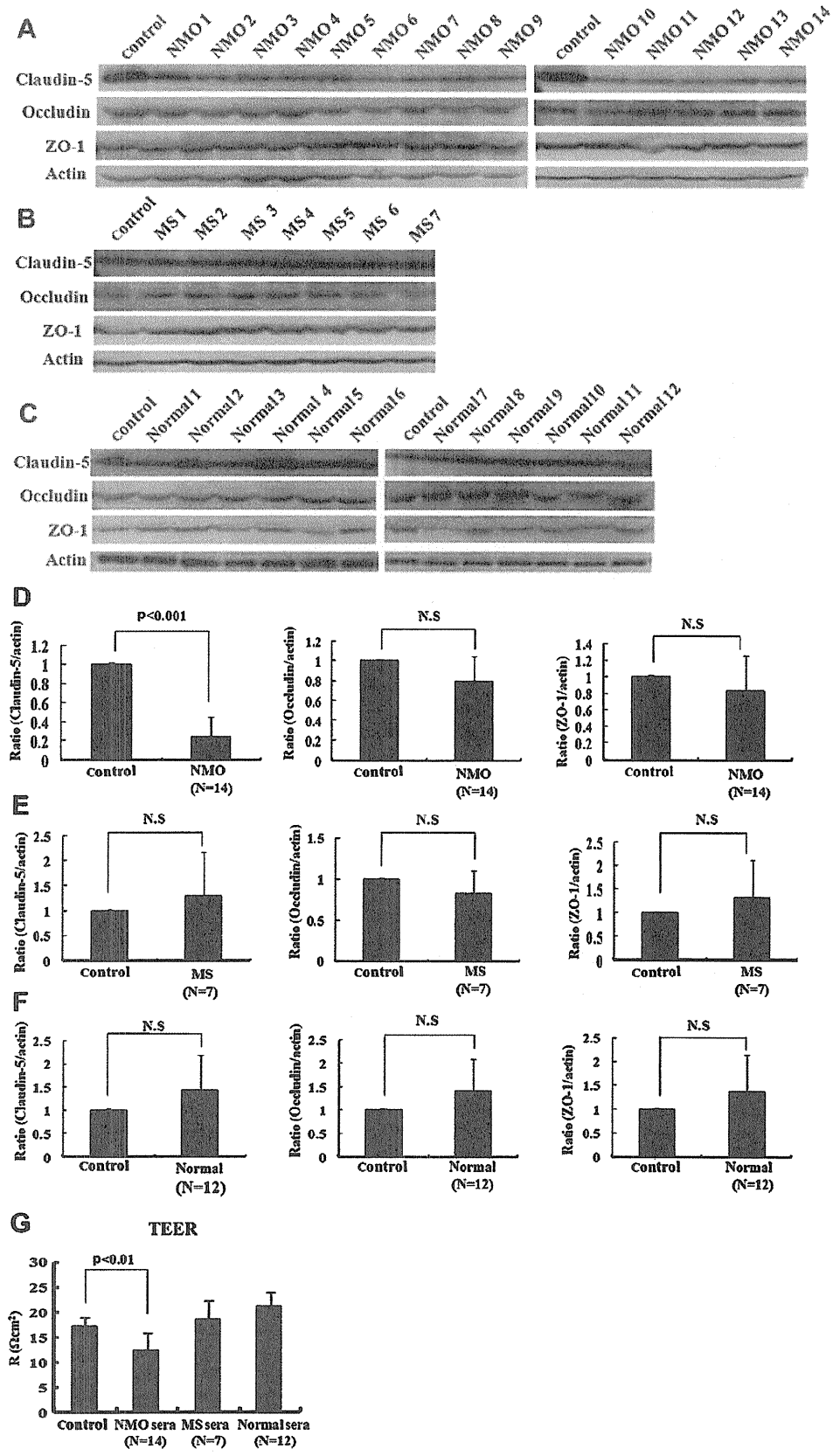

Figure 1 (A–C) Effects of sera on tight junction proteins in human brain microvascular endothelial cells (BMECs) determined by western blot analysis. Changes in claudin-5, occludin and ZO-1 expression in BMECs were determined after exposure to sera from patients with neuromyelitis optica (NMO) or conventional MS (C-MS), or from healthy controls. (D–F) Each bar graph reflects the combined densitometry data from each independent experiment. (D) Expression of claudin-5 protein in BMECs was significantly decreased after exposure to sera from NMO patients (mean±SEM, n=14, $p<0.001$). (E, F) Expression levels of claudin-5 and occludin were not affected by exposure to sera from patients with C-MS (mean±SEM, n=7) or from healthy controls (mean±SEM, n=12). (G) The transendothelial electrical resistance (TEER) value of BMECs was significantly decreased after exposure to NMO sera but was not influenced by exposure to sera from patient with C-MS or from healthy controls. NMO, conditioned medium with 10% serum from an NMO patient diluted with non-conditioned Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS); MS, conditioned medium with a 10% concentration of serum from an MS patient diluted with non-conditioned DMEM containing 10% FBS; Normal, conditioned medium with 10% serum from a healthy control diluted with non-conditioned medium of DMEM containing 10% FBS.