Results

Association of *C8orf13–BLK* rs13277113 with polymyositis/dermatomyositis in the Japanese population

The frequency of the *C8orf13–BLK* rs13277113 A allele was in good agreement with those previously reported for the Japanese population [11,12]. In the present study, the A (risk) allele of rs13277113 was found in 72% of the chromosomes in the polymyositis patients, 76% in the dermatomyositis patients, and 74% in the polymyositis/dermatomyositis patients. All frequencies in the disease subsets were significantly higher than those in the control subjects (64%; $Pcorr = 0.033$, OR 1.32 for polymyositis; $Pcorr = 4.5 \times 10^{-4}$, OR 1.64 for dermatomyositis; and $Pcorr = 3.3 \times 10^{-4}$, OR 1.44 for polymyositis/dermatomyositis). Comparisons of the genotypes showed association of the rs13277113 A allele in a dominant model with dermatomyositis (rs13277113 A/A or A/G genotype; $Pcorr = 0.0011$, OR 4.73). The allele and genotype frequencies are detailed in Table 1.

In the sub-analysis of 138 patients, comprising 46 with polymyositis and 92 with dermatomyositis, the complication of ILD was observed in 46.5% of the polymyositis patients and 66.3% of the dermatomyositis patients; in the combined cohort, 59.8% had ILD. The rs13277113A frequency was equal between patients with ILD (0.75) and those without (0.75). Of the 138 polymyositis/dermatomyositis patients recruited from TWMU, 20.4% were positive for the anti-Jo-1 antibody. The rs13277113A frequency was not statistically significantly different between anti-Jo-1 antibody-positive and antibody-negative patients (0.73 vs. 0.75, respectively). Therefore, no association was found between the rs13277113 polymorphism and the ILD disease phenotype or anti-Jo-1 antibody positivity.

Additive effects of *C8orf13–BLK* and *STAT4*

An additive effect of both risk alleles (the *C8orf13–BLK* rs13277113A allele and the *STAT4* rs7574865 T allele) on susceptibility to polymyositis, dermatomyositis, and polymyositis/dermatomyositis was observed (Table 2).

The OR for polymyositis patients carrying four risk alleles was 2.47 (95% CI 1.40–4.35), using individuals with 0 or 1 allele as a reference. The ORs for dermatomyositis gradually increased: 1.71 (95% CI 1.09–2.57) for carriers of two risk alleles, 2.18 (95% CI 1.36–3.48) for carriers of three risk alleles, and 3.07 (95% CI 1.57–6.02) for carriers of four risk alleles. The ORs for the polymyositis/dermatomyositis patients also gradually increased: 1.64 (95% CI 1.17–2.29) for carriers of three risk alleles and 2.67 (95% CI 1.61–4.42) for carriers of four risk alleles. Therefore, additive effects of *C8orf13–BLK* and *STAT4* were observed, most notably in dermatomyositis.

Discussion

IIMs are clinically and serologically heterogeneous disorders. To date, the genetic basis of IIMs appears to involve at least two major components, viz., HLA regions and non-HLA risk genes common to other autoimmune diseases. The HLA region is associated with overall IIMs susceptibility particularly in Caucasians, in whom the HLA8.1 ancestral haplotype containing DRB1*0301 allele is prevalent, and is tightly linked to production of myositis-specific autoantibodies (MSAs) [2]. However, the association between the HLA region and IIMs is lost in Mexican-American and Korean populations [17]. In the Japanese population, in which the DRB1*0301 allele is rare (0.1–0.2% of the population), DRB1*0803 is weakly associated with susceptibility to IIMs and carriage of anti-aminoacyl-tRNA synthetases (ARS) autoantibodies [18]. Therefore, it seems to be likely that the HLA region is associated with IIM susceptibility to different degrees in different ethnicities, and that it is tightly associated with MSA production. On the other hand, non-HLA risk genes that encode the immune response or cell signaling regulatory proteins are involved in the susceptibility to IIMs, regardless of the presence or not of MSA [2,4,5,19]. Since such risk genes outside of the HLA region are common to other autoimmune diseases, IIMs are likely to share genetic etiology with other autoimmune diseases.

This study presents an association between polymyositis/dermatomyositis and *C8orf13–BLK* rs13277113A in the Japanese population. While preparing this manuscript, data of a GWAS on dermatomyositis in adults and juveniles of European ancestry (n = 1178) were published [19]. According to that study, *BLK* rs2736340 was identified as one of the risk genes for adult and juvenile dermatomyositis in Europeans after screening of 141 non-MHC SNPs that had previously been associated with autoimmune diseases [19]. Because both *BLK* rs2736340 and rs13277113, which were investigated in the present study, are in complete linkage disequilibrium, the risk haplotype identified by GWAS and by the present study are identical. The present Japanese case–control study, as a result, replicated the study of the European GWAS study. To date, few susceptibility genes for IIMs have been

Table 1. Association between *C8orf13–BLK* rs13277113 and polymyositis/dermatomyositis.

Subjects (n)	PM (283)	DM (194)	PM+DM (477)	controls (656)
A allele (frequency)	407 (0.72)	295 (0.76)	702 (0.74)	865 (0.65)
allelic association				
OR (95%CI)	1.32 (1.06–1.64)	1.64 (1.26–2.12)	1.44 (1.19–1.72)	Referent
P	0.011	1.5×10^{-4}	1.1×10^{-4}	-
Corrected *P*	0.033	4.5×10^{-4}	3.3×10^{-4}	-
A/A+A/G (frequency)	262 (0.92)	189 (0.97)	451 (0.94)	583 (0.89)
genotype association				
OR (95%CI)	1.56 (0.94–2.59)	4.73 (1.88–11.9)	2.17 (1.37–3.46)	Referent
P	N.S.	3.6×10^{-4}	8.8×10^{-4}	-
Corrected *P*	N.S.	0.0011	0.0026	-

OR: Odds ratio, CI: confidence interval, PM: polymyositis, DM: dermatomyositis, N.S.: not significant.

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Table 2. A cumulative effect of risk allele number (*C8orf13–BLK* rs13277113A and *STAT4* rs7574865T) on susceptibility to polymyositis, dermatomyositis, and polymyositis/dermatomyositis.

No. of risk alleles	PM (283)		DM (194)		PM+DM (477)	
	OR (95%CI)	*P*	OR (95%CI)	*P*	OR (95%CI)	*P*
0+1	Referent	-	Referent	-	Referent	-
2	1.12 (0.78–1.62)	N.S.	1.71 (1.09–2.57)	0.017	1.34 (0.98–1.83)	N.S.
3	1.37 (0.91–2.03)	N.S.	2.18 (1.36–3.48)	1.8×10^{-3}	1.64 (1.17–2.29)	3.8×10^{-3}
4	2.47 (1.40–4.35)	1.7×10^{-3}	3.07 (1.57–6.02)	1.1×10^{-3}	2.67 (1.61–4.42)	1.4×10^{-4}

OR: Odds ratio, CI: confidence interval, PM: polymyositis, DM: dermatomyositis, N.S.: not significant.
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replicated, except for the HLA 8.1 haplotype in Caucasians, probably due to the different risk allele frequencies in different ethnicities, relatively low disease prevalence, and disease heterogeneity. The present data highlighted the strong contribution of *BLK* to polymyositis/dermatomyositis susceptibility, irrespective of ethnicity.

Accumulating evidence has shown that *BLK* is strongly involved in the development of a wide variety of autoimmune diseases [9–14]. However, it remains unclear how an autoimmune-risk variant within *C8orf13–BLK* influences Blk protein expression, results in altered B cell signaling. Although a risk variant in *C8orf13–BLK* reduces *BLK* mRNA transcript expression in a B cell lymphoblastoid cell line [9], it is unclear whether the variant affects protein expression. However, a recent report showed that the risk variant reduced Blk protein expression in B cells obtained from umbilical cord blood, although not in adult B cell subsets [20]. Reduced Blk expression in the early stage of B cell development may influence B cell receptor signaling, resulting in selection of autoimmune-prone B cells. *Blk*-knockout mice as well as $Blk^{+/-}$ mice exhibited an autoimmune phenotype, with a high titer of anti-nuclear antibody production compared with wild-type mice [21]. B cells are strongly involved in the humoral immune response, particularly as it pertains to autoantibody production.

Therefore, the idea that a risk allele of *C8orf13–BLK* is associated with autoantibody production seems to be reasonable. In the present sub-analysis, however, no increase was observed in the frequency of rs13277113A allele carriers in the anti-Jo-1 antibody-positive group of patients. Interestingly, similar results were previously obtained in SLE patients in whom *BLK* risk loci were not found to be associated with anti-DNA antibody production, although this gene increased disease susceptibility overall [22]. In human $CD4^+$ cells, SNP-associated regulation of *BLK* expression has been found [23]. Therefore, although the mechanism underlying the triggering of autoimmune diseases by a *C8orf13–BLK* risk variant remains unknown, it may influence the overall immune response, including auto-reactive B cell selection or T cell function, resulting in altered individual immune response.

We have previously reported *STAT4* rs7574865 is associated with susceptibility to polymyositis/dermatomyositis in Japanese [5]. STAT-4 is a transcription factor that transduces IL-12, IL-23-, and type-1 interferon-mediated signals into Th1 and Th17 differentiation, monocyte activation, and interferon-gamma production [24]. Among many autoimmune disease-related genes, *STAT4* [25,26], *C8orf13–BLK* [11,12], as well as interferon regulatory factor 5 (*IRF5*) [27] seem to be the most representative susceptibility genes in the Japanese population. In particular, the genetic contribution of *C8orf13–BLK* [11], and to a lesser extent, of *STAT4* [25], are greater in the Japanese population compared with the Caucasian population, due to the high prevalence of the risk gene. Although each risk gene has a relatively low OR for disease susceptibility, the carriage of more risk alleles, in several risk genes, appears to increase the risk for disease susceptibility. Such cumulative associations have been shown in other autoimmune diseases [28], and now also here, by the discovery of the additive effect of alleles in *C8orf13–BLK* and *STAT4* in increasing the risk for polymyositis/dermatomyositis.

The major limitation of the present study was the paucity of association studies in clinical subsets, including serological phenotypes. However, despite the rarity of these diseases, we obtained a large sample size, which provided sufficient statistical power for this case–control study. We identified a susceptibility gene, *C8orf13–BLK*, for polymyositis/dermatomyositis. Both *C8orf13–BLK* and *STAT4* additively increased polymyositis/dermatomyositis susceptibility in the Japanese population.

Key messages

- The *C8orf13–BLK* rs13277113A allele is associated with Japanese polymyositis/dermatomyositis.
- *C8orf13–BLK* rs13277113A and *STAT4* rs7574865T exert additive effects in polymyositis/dermatomyositis susceptibility.

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Author Contributions

Conceived and designed the experiments: TS YK IN HY. Performed the experiments: TS KG YH TG TF. Analyzed the data: TS YK. Contributed reagents/materials/analysis tools: KG YH TG TF IN. Wrote the paper: TS YK.

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BRIEF REPORT

Autoantibodies to DNA Mismatch Repair Enzymes in Polymyositis/Dermatomyositis and Other Autoimmune Diseases: A Possible Marker of Favorable Prognosis

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Objective. **Myositis-specific autoantibodies (MSAs) are useful tools for identifying clinical subsets of patients with idiopathic inflammatory myopathies (IIMs). There have been few reports on antibodies to some DNA mismatch repair enzymes (MMREs) in patients with IIMs. This study was undertaken to determine the frequencies and clinical associations of antibodies to 7 types of MMREs (MLH1, MLH3, MSH2, MSH3, MSH6, PMS1, and PMS2) in patients with IIMs and other systemic autoimmune diseases.**

Methods. **Clinical data and serum samples were collected from 239 Japanese patients with IIMs (147 with adult dermatomyositis, 13 with juvenile dermatomyositis, 57 with polymyositis, and 22 with myositis overlap syndrome). One hundred patients with other diseases, including 40 with systemic lupus erythematosus (SLE), were assessed as disease controls. The presence of anti-MMRE antibodies in serum was examined by immunoprecipitation, enzyme-linked immunosorbent assay, and immunoprecipitation/Western blotting.**

Results. **Anti-MMRE antibodies were found in 15 patients with IIMs and 3 patients with SLE. They were restricted to MLH1, PMS1, MSH2, and PMS2, with simultaneous positivity for more than one of these antibodies occurring in some patients. Nine IIM patients with anti-MMREs also had other MSAs and their associated clinical features. All patients with anti-MMREs were still living at the time of the present analysis.**

Conclusion. **Anti-MMRE antibodies, which often coexist with other MSAs, may be serologic markers for good prognosis in IIMs.**

Idiopathic inflammatory myopathies (IIMs) are systemic autoimmune diseases that mainly affect muscle and/or skin. Various myositis-specific autoantibodies (MSAs) and myositis-associated autoantibodies (MAAs) have been described (1). MAAs have been reported in relation to myositis in overlap syndromes with other autoimmune diseases. In contrast, MSAs are exclusive to myositis, and ≥2 MSAs rarely coexist in a single patient.

DNA mismatch repair is one of several DNA repair pathways conserved from bacteria to humans. The primary function of mismatch repair is to eliminate the mismatch of base–base insertions and deletions that appear as a consequence of DNA polymerase errors during DNA synthesis. In humans, there are 2 sets of mismatch repair enzymes (MMREs), corresponding to homologs of the bacterial MutS and MutL systems (2). The human MutS homologs are MSH2, MSH3, and MSH6, and human MutL homologs include MLH1, MLH3, PMS1, and PMS2.

A 2001 report described the presence of autoantibodies to PMS1 in patients with IIM (3). Autoantibodies to PMS2 and MLH1 were also present in some patients. In 2005, anti-PMS1 and anti-MSH2 antibodies were found in Japanese patients with IIMs (4). In the present study, we evaluated the frequencies and clinical implications of autoantibodies to the 7 types of MMREs in patients with IIM and other autoimmune diseases.

PATIENTS AND METHODS

Patients. Serum samples from 239 Japanese patients (56 male, 183 female) with IIM (147 with adult dermatomyo-

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sitis [DM], 13 with juvenile DM, 57 with polymyositis [PM], and 22 with myositis overlap syndrome) were analyzed for this study. The sera were from 144 patients seen at Nagoya University Hospital between 1994 and 2013 and 95 patients seen at Kyoto University Hospital between 2004 and 2013. They were obtained at the time of diagnosis (except for 2 samples obtained at a later time point from patients with juvenile DM) and were from consecutive patients in most cases (samples from some consecutively diagnosed patients were not available for study). One hundred patients with other autoimmune diseases (40 with systemic lupus erythematosus [SLE], 20 with systemic sclerosis [SSc], 20 with rheumatoid arthritis [RA], and 20 with Sjögren's syndrome [SS]) were assessed as disease controls. All 57 patients with PM and 95 of the patients with adult DM fulfilled the Bohan and Peter criteria (5); 52 patients fulfilled the Sontheimer criteria for clinically amyopathic DM (CADM) (6). The patients with SLE, RA, and SSc met the respective American College of Rheumatology classification criteria for these diseases (7–9). SSc was classified as diffuse cutaneous or limited cutaneous according to the criteria of LeRoy et al (10). SS was diagnosed based on Japanese diagnostic criteria (11). Clinical information was collected retrospectively by medical chart review. The study was conducted with the approval of the ethics committees of the Nagoya University Graduate School of Medical Science and the Kyoto University Graduate School of Medical Science.

Laboratory tests and serologic assays. Serum samples were screened for antibodies against SSA, SSB, U1 RNP, Sm, CENP-B, and double-stranded DNA (dsDNA) using commercial enzyme-linked immunosorbent assay (ELISA) kits (MBL). In addition, anti–Mi-2, anti–transcription intermediary factor 1γ (anti–TIF-1γ), anti–melanoma differentiation–associated protein 5 (anti–MDA-5), anti–nuclear matrix protein 2 (anti–NXP-2), and anti–aminoacyl–transfer RNA synthetase (anti-aaRS) antibodies were investigated by protein and RNA immunoprecipitation (12) and/or immunoprecipitation with recombinant protein (13).

Immunoprecipitation and ELISA using recombinant protein. The full-length complementary DNA (cDNA) clones of 7 human MMREs (Flexi ORF Clone) were purchased from Promega. Biotinylated recombinant protein was produced from the cDNA, using a T7 Quick Coupled Transcription/Translation System (Promega) according to our previously described protocol (13). Briefly, 800 μl TnT Quick Master Mix, 20 μl 1 m*M* methionine, 30 μl biotin-lysyl–transfer RNA, 120 μl water, and 30 μl DNA (1 μg/μl) were mixed and then incubated for 60 minutes at 30°C. Immunoprecipitation was performed using in vitro translation and transcription (TnT) products as previously described (13).

Anti-MMRE antibodies were also tested by antigen-capture ELISA according to our previously described methods (14). Briefly, a 96-well Immobilizer Streptavidin Plate (Thermo Scientific Nunc) was incubated with 1 μl/well of TnT reaction mixture including biotinylated recombinant protein. Wells were then incubated with sera (1:1,000 dilution) and probed with horseradish peroxidase–conjugated anti-human IgG antibody (1:30,000 dilution; Dako). After incubation with SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo Scientific Pierce), relative luminescence units (RLU) were determined using a GloMax-Multi Detection System (Promega). Each serum sample was tested in duplicate, and the mean RLU minus background was used for data analysis. The RLU of the samples was converted into units using a standard curve created with a prototype positive serum.

Immunoprecipitation/Western blotting. Immunoprecipitation assays were performed using extracts of the leukemia cell line K562 as previously described (12), with minor modifications. Patient serum (10 μl) was bound to 10 μl of protein G–Sepharose Fast Flow (GE Healthcare Japan) in 500 μl of immunoprecipitation buffer (10 m*M* Tris HCl [pH 8.0], 500 m*M* NaCl, 0.1% Nonidet P40) and incubated for 2 hours at 4°C, followed by washing 5 times with immunoprecipitation buffer. Antibody-coated Sepharose beads were mixed with 100 μl K562 cell extracts derived from 10^6 cells and rotated at 4°C for 2 hours. After 5 washes, the beads were resuspended in sodium dodecyl sulfate (SDS) sample buffer, and samples were fractionated by SDS–polyacrylamide gel electrophoresis followed by Western blotting. Polyclonal antibodies to MLH1, MSH2, PMS1, and PMS2 were purchased from Santa Cruz Biotechnology.

Statistical analysis. Statistical significance was assessed by Fisher's exact test, Mann-Whitney U test, or log rank test, as appropriate. Data were evaluated using SPSS Statistics (IBM). *P* values less than 0.05 were considered significant.

RESULTS

Detection of anti-MMRE antibodies in patients with IIM and other autoimmune diseases. Serum samples from 239 patients with IIM were screened for anti-MMREs using immunoprecipitation with recombinant proteins. PMS1, MLH1, MSH2, and PMS2 recombinants were immunoprecipitated by 10, 9, 3, and 2 sera, respectively, as determined by TnT immunoprecipitation (Figure 1). They were confirmed to react with the corresponding proteins, since the precipitates were recognized by polyclonal antibodies to these proteins in Western blotting (Figure 1). MLH3, MSH3, and MSH6 were not reactive with any sera from the IIM patients, although their recombinants were produced (Figure 1).

In all of the IIM sera, the presence of each of the 7 anti-MMRE antibodies was also examined by ELISA. With positivity defined as an RLU value more than 5 SD above the mean in 20 healthy controls, these analyses demonstrated that all of the sera that were positive for antibodies to PMS1, MLH1, MSH2, and PMS2 by immunoprecipitation were positive by ELISA as well (data available from the corresponding author upon request). As in the immunoprecipitation analyses, no sera were found by ELISA to be positive for anti-MLH3, anti-MSH3, or anti-MSH6 antibodies.

Positivity for antibodies to PMS1, MLH1, MSH2, and PMS2 was also assessed by ELISA in the sera of disease controls and healthy controls (data available from the corresponding author upon request). The only

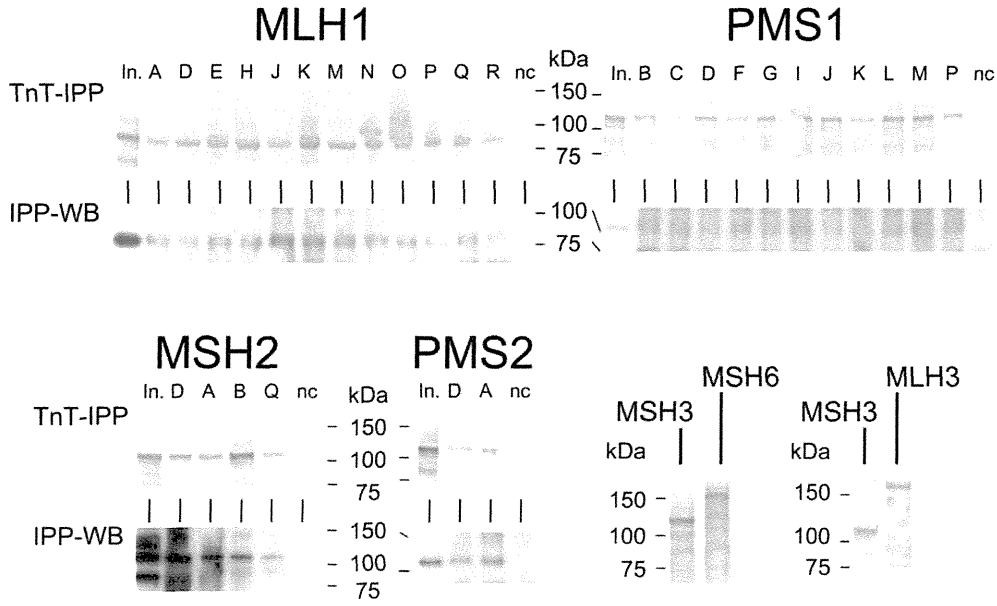

Figure 1. Detection of antibodies to DNA mismatch repair enzyme (anti-MMRE). In translation and transcription immunoprecipitation (TnT-IPP) experiments, biotinylated recombinant MLH1, PMS1, MSH2, and PMS2 were assessed by immunoprecipitation. Recombinant proteins were subjected to 4–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting with streptavidin–alkaline phosphatase and substrate. In immunoprecipitation/Western blotting (WB) experiments, immunoprecipitates from K562 cell extracts with human sera were probed with polyclonal antibodies to MLH1, PMS1, MSH2, and PMS2. The input (In.) was half the dose (or a full dose for immunoprecipitation/Western blotting of MLH1), of the biotinylated proteins or cell extract used for the immunoprecipitation assay. Biotinylated recombinant MSH3, MSH6, and MLH3 were also subjected to 4–20% SDS-PAGE and analyzed by immunoblotting with streptavidin–alkaline phosphatase and substrate; no serum samples immunoprecipitated these recombinants. Lanes A–R correspond to anti-MMRE–positive patients shown in Table 1. nc = normal control serum.

positive results in these assays were found in 3 sera from patients with SLE. All 3 SLE sera were reactive with MLH1; 1 was additionally reactive with PMS1, and another was additionally reactive with MSH2. The presence of antibodies to MLH1, PMS1, and MSH2 in these 3 patients with SLE was confirmed by immunoprecipitation/Western blotting (Figure 1).

Longitudinal study of anti-MMRE antibodies coexisting in individual patients. Among a total of 18 IIM or SLE patients with anti-MMRE, 8 were positive for at least 2 types of anti-MMRE antibodies (Table 1). Patterns of reactivity with 4 MMREs (MLH1, PMS1, MSH2, and PMS2) and their combinations were heterogeneous among patients and were not associated with the specific disease or disease subset (data available from the corresponding author upon request). The coexistence of anti-MLH1 and anti-PMS1 antibodies was found most frequently (5 patients). All patients who were positive for anti-MSH2 and/or anti-PMS2 were also positive for anti-MLH1 and/or anti-PMS1.

To further investigate the associations of antibodies to different MMREs, we obtained longitudinal serum samples from 7 patients who were positive for >1 type of anti-MMRE and examined antibody titers by ELISA (data available from the corresponding author upon request). In patient D, who was positive for 4 different anti-MMREs, titers of all 4 decreased similarly over time. Titers of anti-MLH1 changed in parallel to those of anti-PMS1 in patients J, K, M, and P and in parallel to those of anti-MSH2 in patient Q. In patient B, titers of anti-PMS1 changed similarly to titers of anti-MSH2.

Clinical and laboratory profiles of patients with anti-MMRE antibodies. Of the 239 patients with IIM, 15 were positive for at least 1 anti-MMRE antibody: 5 (5.3%) of 95 adults with DM, 3 (5.3%) of 57 adults with PM, 2 (3.8%) of 52 adults with CADM, 2 (15.4%) of 13 juvenile patients with DM, and 3 (13.6%) of 22 adults with myositis overlap (Table 1). The antibody frequency was higher among female IIM patients (15 of 183) than among male patients (0 of 56) ($P < 0.026$). Muscle symptoms and arthralgia were seen in 12 and 10 patients, respectively, while internal malignancy was not found. Among adult patients with DM including CADM, anti-

Table 1. Clinical and laboratory features of the IIM patients and disease control patients who were found to be positive for antibodies to MMRE*

	Patient																	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
Age, years	48	56	70	22	19	28	40	30	47	25	15	4	40	44	63	38	15	28
Sex	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F	F
Diagnosis	PM	PM	PM	DM	DM	DM	DM	DM	CADM	CADM	JDM	JDM	PM–SSc	PM–SSc	PM–SLE	SLE	SLE	SLE
Anti-MLH1	+	–	–	+	+	–	–	+	–	+	+	–	+	+	+	+	+	+
Anti-PMS1	–	+	+	+	–	+	+	–	+	+	+	+	+	–	–	+	–	–
Anti-PMS2	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Anti-MSH2	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Other autoantibodies	–	Jo-1, SSA	–	–	–	TIF-1γ	MDA-5	MDA-5	PL-7	MDA-5	TIF-1γ	TIF-1γ	–	CENP-B	PL-12, SSA, dsDNA	U1 RNP, SSA, dsDNA	U1 RNP, Sm, SSA, dsDNA	U1 RNP, SSA
Muscle symptoms†	+	+	+	+	+	+	+	+	–	–	+	–	+	+	+	+	–	–
Highest CK level, IU/liter	339	393	3,405	1,263	150	6,554	209	220	202	101	130	65	1,016	195	437	252	87	95
Gottron's sign	–	–	–	+	–	+	+	+	+	+	+	+	–	–	–	–	–	–
Heliotrope rash	–	–	–	–	+	+	–	+	–	+	+	+	–	–	–	–	–	–
Mechanic's hands	–	–	–	–	–	–	–	+	+	–	–	–	–	–	+	–	–	–
Arthralgia	–	+	+	+	+	–	+	+	–	+	–	–	+	+	+	+	+	+
ILD	–	+	+	–	–	–	+	+	+	–	–	–	–	–	+	–	–	–
Malignancy	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

* IIM = idiopathic inflammatory myopathy; MMRE = DNA mismatch repair enzyme; PM = polymyositis; DM = dermatomyositis; CADM = clinically amyopathic DM; JDM = juvenile DM; SSc = systemic sclerosis; SLE = systemic lupus erythematosus; TIF-1γ = transcription intermediary factor 1γ; MDA-5 = melanoma differentiation–associated protein 5; dsDNA = double-stranded DNA; CK = creatine kinase; ILD = interstitial lung disease.
† Muscle weakness and/or myalgia.

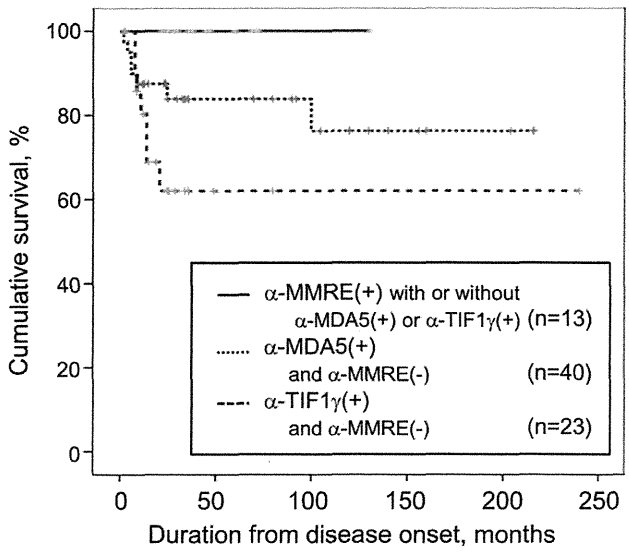

Figure 2. Cumulative survival rates from the time of disease onset in 76 Japanese patients with polymyositis/dermatomyositis (including clinically amyopathic dermatomyositis and myositis overlap syndrome) who were positive for serum anti–mismatch repair enzyme (anti-MMRE), anti–melanoma differentiation–associated protein 5 (anti–MDA-5), or anti–transcription intermediary factor 1γ (anti–TIF-1γ). The analysis did not include anti-MMRE–positive patients with juvenile dermatomyositis or systemic lupus erythematosus, although these patients were also still alive at the time of the analysis. The anti-MMRE–positive group included some patients who were also positive for anti–MDA-5 (n = 3) or anti–TIF-1γ (n = 1); no patients in the other 2 groups were positive for anti-MMRE. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.38866/abstract.

MMRE–positive patients were younger than antibody-negative patients ($P < 0.001$).

MSAs or systemic autoimmune disease–associated autoantibodies were found in 13 of the 18 IIM or SLE patients who were anti-MMRE positive. The prevalences of anti-MMREs in anti–MDA-5–positive patients (3 of 43 [7%]) and in anti–TIF-1γ–positive patients (3 of 30 [10%]) were not significantly higher than the prevalence of anti-MMREs in anti–Jo-1–positive patients (1 of 22 [4.5%]). Anti-aaRS and anti–MDA-5 antibodies were found in 3 patients and 2 patients, respectively, all of whom had interstitial lung disease. Three patients with anti–TIF1-γ were either juvenile or young adult patients with DM. Patients who were positive for anti–U1 RNP and/or anti-dsDNA were all diagnosed as having SLE. Of the 5 patients who were positive for at least 1 anti-MMRE antibody but for no other autoantibodies, all had IIMs.

Since all of the patients with anti-MMREs were still living at the time of the present analysis, we compared the survival rates among 3 groups: 1) patients who were positive for anti-MMRE with or without anti–MDA-5 or anti–TIF-1γ (excluding those with SLE or juvenile DM), 2) patients who were positive for anti–MDA-5 and negative for anti-MMRE, and 3) patients who were positive for anti–TIF-1γ and negative for anti-MMRE (Figure 2). Cumulative survival rates were lower among patients with anti–MDA-5 and in patients with anti–TIF-1γ compared to patients with anti-MMRE ($P = 0.136$ [not significant] and $P = 0.016$, respectively).

DISCUSSION

The present study is the first in which autoantibodies to 7 different types of MMREs were investigated. There are 2 previous reports describing the detection of anti-MMRE antibodies in patients with IIMs (3,4). Casciola-Rosen reported 6 patients with IIM and anti-MMRE positivity (3); anti-PMS1, anti-MLH1, and anti-PMS2 antibodies were found in 4, 3, and 2 patients, respectively. Japanese investigators initially detected anti-PMS1 and anti-MSH2 antibodies in a patient with pancreatic cancer (4). Subsequently, they detected anti-PMS1 in 13.5% of pancreatic cancer patients and in 6.7% of PM/DM patients by immunoprecipitation with TnT protein, and anti-MSH2 in 8.1% of pancreatic cancer patients and in 4.9% of PM/DM patients by immunoblotting with bacterial recombinant protein. Although detailed clinical information on the PM/DM patients was not reported, it was noted that 1 of the anti-PMS1–positive PM/DM patients had breast cancer.

In our study, anti-MLH1, anti-PMS1, anti-MSH2, and anti-PMS2 antibodies were detected, whereas no subject was positive for anti-MSH2, anti-HSH6, or anti-MLH3. Interestingly, nearly half of the antibody-positive patients (8 of 18) had multiple anti-MMRE antibodies, with the titers of the different antibodies changing in parallel within individual patients. We performed homology searches, using Pearson's online *lalign* program (http://www.ch.embnet.org/software/LALIGN_form.html), among amino acid structures of MSH1, PMS1, PMS2, and MSH2. These revealed no significantly homologous regions in long stretches among the 4 proteins. However, we found a highly homologous sequence with a 12–amino acid stretch ($T^{Y}/_{F}GFRGEAL^{A}/G/_{S}S^{I}/_{L}$) at the N-terminus of MLH1, PMS1, and PMS2, which MSH2 does not have. Thus, we cannot completely exclude the possibility of cross-reactivities among MLH1, PMS1, and/or PMS2.

Since only patients with IIM (n = 5) were positive for anti-MMRE but not for other autoantibodies, anti-MMREs can be considered as MAAs. The clinical features of the other 13 patients were closely associated with coexistent autoantibodies. In a previous study, 1 DM patient also had anti–Mi-2 and 1 PM patient had serologic evidence of SLE (autoantibodies to poly[ADP-ribose] polymerase and to catalytic subunit of DNA-dependent protein kinase) (3). Several MSAs have been discovered in recent years, and some of these autoantibodies may have been concomitantly present, but not tested for, in IIM patients who were found to be positive for anti-MMRE before the other MSAs were identified.

All of the IIM patients in whom anti-MMREs had been identified were still living at the time of the present analysis. The cumulative survival rate among these patients was significantly better than among patients with anti–TIF-1γ, which is a serologic marker for cancer-associated DM (1,13); the increased survival among patients with anti-MMREs compared to those with anti–MDA-5, which was originally defined as a serologic marker for CADM complicated by rapidly progressive interstitial lung disease (1,12,13), was not significant. There are conflicting reports regarding outcomes among anti–MDA-5 antibody–positive patients with IIM (15), but recent therapeutic advances are resulting in improved survival for this group (Muro Y, et al: unpublished observations).

In summary, anti-MMREs are considered to be myositis-associated antibodies, but clinical subsets are strongly influenced by coexistent autoantibodies. Inclusion of a larger number of disease control patients in the present study would likely have improved our ability to assess this in greater detail. Further study is needed to investigate whether the antibodies described herein might have prognostic, in addition to diagnostic, value.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Muro had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Muro, Nakashima, Mimori, Akiyama.
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Establishment of an ELISA to detect anti-glycyl-tRNA synthetase antibody (anti-EJ), a serological marker of dermatomyositis/polymyositis and interstitial lung disease

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ABSTRACT

Background: The aminoacyl transfer RNA synthetases (ARSs) are a group of enzymes that charge amino acids to the cognate transfer RNA during the translation process. Previous reports demonstrated autoantibodies to 8 different ARS. Although anti-glycyl-tRNA synthetase antibodies (anti-EJ) are mainly found in patients with inflammatory myopathy, information on their clinical significances is limited, partly due to a lack of commercially available tests.

Methods: We developed an ELISA and immunoprecipitation method by using recombinant EJ protein to detect the anti-EJ of 453 patients with various autoimmune connective tissue diseases (ACTDs). We also studied the influence of 3 cytokines—IL-1β, IFN-γ and IFN-α—on the level of EJ mRNA and protein expressed by human fetal lung fibroblasts.

Results: Five patients were positive for anti-EJ. Although 3 of these patients had dermatomyositis/polymyositis, the other 2 patients did not have myositis. The three patients with high levels of anti-EJ antibodies in ELISA were complicated with interstitial lung disease. There was no significant change in the level of EJ protein expressed by human fetal lung fibroblasts stimulated by the cytokines.

Conclusion: We developed an ELISA to detect anti-EJ by using recombinant protein. This easy-to-use ELISA could help clarify the clinical significance of anti-EJ in ACTDs.

1. Introduction

Idiopathic inflammatory myopathies (IIMs) are systemic autoimmune inflammatory diseases that mainly involve muscle and/or cutaneous tissues. Various myositis-specific autoantibodies (MSAs) and myositis-associated autoantibodies (MAAs) have been reported [1,2]. In many cases, the MAAs have been reported in relation to overlap syndrome in autoimmune connective tissue diseases (ACTDs). In contrast, MSAs are exclusive and two different MSAs rarely coexist in a patient [3,4].

MSAs are highly consistent with the clinical subtype and could be effectively predictive factors for disease course, therapy response and prognosis [1,5]. For example, anti-melanoma differentiation-associated protein 5 (MDA5) antibodies have been demonstrated to correlate with rapid progressive interstitial lung disease (ILD), and anti-transcriptional intermediary factor 1 (TIF1)-γ antibodies are known to be closely associated with internal malignancies [6]. Autoantibodies to aminoacyl transfer RNA synthetases (ARSs) have been reported to be associated with myositis, fever and ILD not only in dermatomyositis/polymyositis (DM/PM) but also in other ACTDs [5,7].

ARSs, which exist in whole body cells ubiquitously, are a group of enzymes that charge amino acids to the cognate transfer RNA during the translation process [8]. To date, autoantibodies to 8 different ARSs have been reported, including anti-Jo-1 antibody, the most well-known anti-ARS, which targets histidinyl-tRNA synthetase protein. These antibodies have been detected by complicated RNA and protein analyses by the technique of immunoprecipitation using silver staining or analyses based on radioisotope to detect RNAs and by the immunoprecipitation assay based on radioisotope to detect proteins [3,9].

Anti-EJ antibody, another anti-ARS, targets glycyl-tRNA synthetase. Recently, a multi-center study reported that more than half of all patients with anti-EJ were diagnosed with classical DM and clinically amyopathic DM (CADM) [9]. Although this result suggests that anti-EJ antibody is an important biomarker especially in dermatologic clinics,

Abbreviations: ARS, aminoacyl transfer RNA synthetase; ACTD, autoimmune connective tissue disease; MDA5, melanoma differentiation-associated protein 5; ILD, interstitial lung disease; TIF1, transcriptional intermediary factor 1; IIM, idiopathic inflammatory myopathy; MSA, myositis-specific autoantibody; MAA, myositis-associated autoantibody; DM, dermatomyositis; PM, polymyositis; CADM, clinically amyopathic dermatomyositis; SLE, systemic lupus erythematosus; lSSc, limited cutaneous systemic sclerosis; dSSc, diffuse cutaneous systemic sclerosis; ACR, American College of Rheumatology; SS, Sjögren's syndrome; TnT, translation and transcription; IPP, immunoprecipitation, IFN, interferon.

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the previous results [9] may be biased due to sampling from many referral centers. In this study, we investigated anti-EJ in serum samples from a single center using an ELISA system that we newly established by using recombinant EJ protein. Moreover, we studied effects of cytokines on the expression of the EJ gene and protein in fetal lung fibroblast cells, since many authors have recently reported that various cytokines, including those of the TNF family, interleukins and interferons, have various effects on the pathogenesis of DM/PM [10,11].

2. Materials and methods

2.1. Patients and sera

From the serum bank of the Department of Dermatology, Nagoya University Hospital, we used sera from 453 Japanese patients with ACTDs: 225 with systemic sclerosis (SSc), 119 with systemic lupus erythematosus (SLE), 85 with DM, 10 with PM and 14 with ILD (Table 1). In our study, the category of DM includes classical DM and CADM. All the classical DM and PM patients fulfilled the Bohan and Peter's criteria [12,13]. The CADM patients fulfilled Sontheimer's criteria [14]. SLE was diagnosed according to the American College of Rheumatology (ACR) criteria [15]. SSc was diagnosed according to the diagnostic criteria [16] established by the Ministry of Health, Labour and Welfare of Japan. SSc patients were divided into two subsets: limited cutaneous SSc and diffuse cutaneous SSc [17]. Patients were diagnosed as having ILD by chest radiography and/or chest computed tomography. The patient's group categorized in ILD includes the 11 patients with ILD alone and the 3 with Sjögren's syndrome (SS)-related ILD. SS was diagnosed based on diagnostic criteria established by the Ministry of Health, Labour and Welfare of Japan [18]. This study was approved by the Ethics Committee of Nagoya University, Graduate School of Medicine, and was conducted according to the principles of the Declaration of Helsinki.

2.2. Protein expression and purification of bacterial recombinant EJ protein

The full-length cDNA clone of human glycyl-tRNA synthetase (GeneBank reference number BC007755), which was purchased from Kazusa DNA Research Institute, was subcloned into the pENTR221 Gateway entry vector (Invitrogen). Then, the cDNA was moved into the pDEST17 Gateway destination vector using Gateway cloning technology. The pDEST17 vector is designed to contain 6xHis-tag in the N-terminal. The vector was transformed into the Rosetta 2 *Escherichia coli* competent cell strain DE3 (Novagen) and the bacterial proteins were harvested after cell culture. The his-tagged protein in the harvested bacterial proteins was purified using Ni Sepharose 6 Fast Flow (GE Healthcare) under denaturing conditions with 6 mol/l urea according to the manufacturer's protocol.

2.3. ELISA with recombinant EJ protein

ELISA was performed as described previously with slight modifications [19,20]. Briefly, wells of microplates (Medisorp; Nunc Roskilde) were coated with purified recombinant EJ protein (0.4 μg/100 μl/well) described as above. The optimal protein concentration used as antigen in ELISA was determined by experiments using various concentrations of antigen and antibodies (data not shown). After overnight incubation at 4 °C and washing, the wells were incubated with 200 μl blocking buffer of 3% bovine serum albumin solution diluted with 0.05% Tween-PBS for 2 h. Patient sera were diluted to 1:100 with T-PBS and applied to the wells as primary antibody and incubated for 1 h at RT. Anti-human IgG antibody conjugated with HRP (Dako) was applied to the wells as the secondary antibody and incubated for 1 h at RT. The development was performed by using 1 Step Ultra TMB (Piece) at 100 μl/well, stopped by 0.5 mol/l sulfuric acid. Each serum sample was measured in duplicate, and the mean optical density (OD) at 450 nm was used for analysis. Twenty sera of healthy controls were measured on every plate. In addition, the OD of each serum sample in uncoated wells was measured as the background level. In order to compare the results for different plates, all ELISA results were transformed into ELISA unit values using the following formula:

$$\text{ELISA unit value} = 100 \times (\text{corrected OD of serum from each patient})/\text{the cutoff OD value}.$$

The corrected OD value was the OD value of each sample minus the background level. The cutoff value was determined for every plate as the mean of the corrected OD values obtained from 20 control sera + 5 SD.

Table 1
Patient groups and anti-EJ antibody frequencies.

Clinical group	Age[a] range, year	Mean age[a] (S.D.) in years	Gender, male: female	Total	α-EJ (%)
SLE	8–76	43 (15)	10:109	119	1 (0.8)
lSSc	26–83	56 (10)	6:112	118	0
dSSc	28–84	53 (14)	16:91	107	0
DM	3–84	49 (18)	24:61	85	2 (2.3)
Classical	3–84	49 (18)	22:44	66	2 (3)
CADM	9–73	50 (18)	2:17	19	0
ILD	24–73	56 (10)	4:10	14	1 (7)
PM	32–72	61 (11)	2:8	10	1 (10)

[a] At the time of sera collection.

2.4. Immunoprecipitation using biotinylated recombinant EJ protein

Biotinylated recombinant EJ protein was produced from pDETST17 vector harboring EJ cDNA constructed as above using an *in vitro* translation and transcription (TnT) system that makes use of rabbit reticulocyte lysate (Promega) according to the manufacturer's protocols. Immunoprecipitation (IPP) was performed as described with slight modifications [6,21,22]. In brief, 4 μl of biotinylated recombinant EJ protein, 10 μl of each serum sample (3 μl in the experiment with polyclonal anti-EJ antibody), 20 μl (bed volume: 10 μl) of protein G sepharose (GE Healthcare) and 0.5% NP40-PBS (IPP buffer) were mixed in one tube (total volume 300 μl) and incubated with moderate agitation for 1 h at RT. After 5 washings with IPP buffer, the sepharose bead-bound proteins were electrophoresed by SDS-PAGE and transferred to Western blotting membranes. Biotinylated proteins were detected by Western blot using Western Blue Substrate (Promega) according to the manufacturer's protocols.

2.5. Cell culture and stimulation by IL-1β, IFN-γ and IFN-α

The normal human fetal lung fibroblast cells (WI-38) (Riken) were cultured in Minimum Essential Medium (Sigma-Aldrich) with 10% fetal bovine serum (Equitech-Bio) and 2 mM L-glutamine at 37 °C under 5% CO_2 atmosphere. The cultured WI-38 cells (1×10^5 cells/well) were seeded into 6-well plates and incubated for 24 h. After washing with PBS, the cultured WI-38 cells were stimulated by cytokines in the culture medium. The 3 kinds of cytokines were used to stimulate the cells: IL-1β (PeproTech), IFN-γ (PeproTech) and IFN α-2a (Prospec). IL-1β, IFN-γ and IFN α-2a were used at the respective concentrations of 10 ng/ml, 15 ng/ml and 1000 IU/ml. Cells were harvested according to the time course at 24, 48, 72 h, and the EJ mRNA and protein levels were analyzed by real-time quantitative PCR (RT-PCR) and Western blot, respectively. The WI-38 cells were harvested using Trypsin-EDTA solution (Sigma-Aldrich) to peel the cells from the plate for RT-PCR. The cells were harvested with IPP buffer and a scraper was used to peel off the cells physically for Western blot.

2.6. Real-time quantitative PCR

mRNAs of the harvested cultured cells were extracted by using the RNeasy Mini Kit (Qiagen) according to the manufacture's protocols. The mRNA levels were analyzed using two-step TaqMan probe (Hokkaido System Science Co., Ltd) RT-PCR according to the manufacturer's protocols. The primers and probe of EJ are as follows: forward, 5′-AGGAGGTTTTTCTATGATC-3′; reverse, 5′-TTGT TCTTCAAAGCACAG-3′; TaqMan, 5′-TCATACAGACCACTAACACCTC CA-3′. Reactions were run on a Sequence Detector System LightCycler 480 (Roche Diagnostics).

2.7. Western blot

The harvested cells were agitated slightly and incubated in IPP buffer for 30 min at 4 °C. After centrifugation at 14,000 rpm at 4 °C, the liquid phase was harvested and electrophoresed by SDS-PAGE and transferred to Western blotting membranes. The membranes were incubated in the blocking buffer (5% skim milk in 0.05% Tween20-PBS) overnight at 4 °C. Then, these membranes were reacted with anti-EJ antibody (dilution 1:1000, Abnova) and anti-β-actin antibody (dilution 1:50,000, Sigma-Aldrich) which were used as first antibodies for 1 h at RT. The membranes were reacted with HRP-conjugated rabbit immunized anti-mouse immunoglobulin antibody (dilution 1:1000, Dako) which was used as the second antibody for 1 h at RT. The signal was developed using ECL Plus Western Blotting Detection System (GE Healthcare) and detected by ImageQuant LAS4000 (GE Healthcare).

2.8. Statistical analysis

The Student's *t*-test was used to test the significant difference of the mRNA expression levels between the cytokine-stimulated group and the untreated group.

3. Results

3.1. Measurement of the anti-EJ antibody by ELISA

We performed the ELISA using the bacterial recombinant EJ protein to screen the anti-EJ antibody. The sera whose ELISA units exceeded 100 were regarded as ELISA-positive, with the ELISA units calculated based on 20 healthy control sera, as mentioned above. In this study, we detected 5 anti-EJ antibody-positive patients from the 458 patients (Fig. 1). Two were patients with DM, 1 was with PM, 1 was with SS-related ILD and the other was with SLE. We detected no anti-EJ antibody-positive patients with SSc.

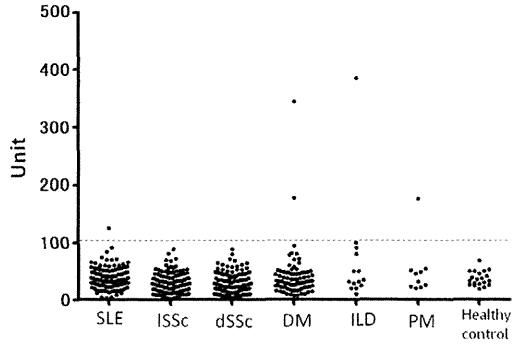

Fig. 1. Measurement of anti-EJ antibodies by ELISA. A total of 453 serum samples from patients with various diseases and 20 healthy control sera were examined. Anti-EJ antibodies were found in 1 patient with SLE, 2 with DM, 1 with ILD and 1 with PM. The broken line indicates the cutoff value (100 units), calculated as the mean OD value of 20 healthy control serum samples + 5 standard deviations.

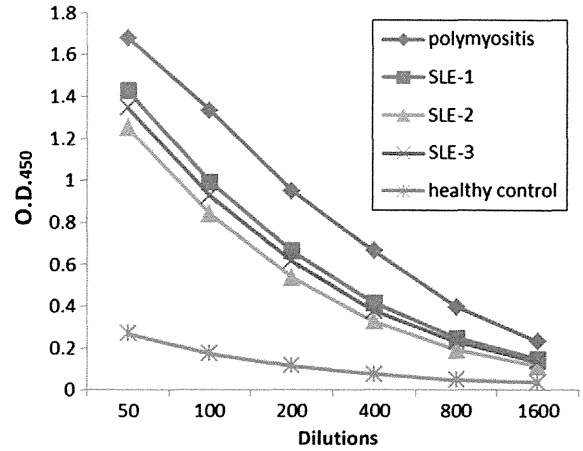

Fig. 2. Titration study of SLE sera in ELISA. Three longitudinal sera from a patient with SLE who was positive for anti-EJ by ELISA (Fig. 1) were diluted 50-fold to 1600-fold. PM: patient #4 (Table 2); SLE-1: serum obtained at the disease onset; SLE-2: serum obtained 6 months after SLE-1; SLE-3: serum obtained 6 months after SLE-2.

Since anti-ARS antibodies are rarely found in patients with SLE [9], we performed the ELISA using the serially diluted longitudinal sera from the patient with SLE who was positive for anti-EJ antibody by ELISA. All three longitudinal sera obtained at every half a year from disease onset showed good linearity of ELISA (Fig. 2).

3.2. Immunoprecipitation using biotinylated recombinant EJ protein

To confirm the results of ELISA, we performed the IPP of the sera determined as anti-EJ-positive by ELISA. We used the biotinylated recombinant EJ protein of the TnT product in the IPP (Fig. 3, lane input). In all 5 patients' sera, which were positive in ELISA, sera of the 2 patients with DM (Fig. 3, lanes 1 and 2), 1 with PM (Fig. 3, lane 3) and 1 with SS-ILD (Fig. 3, lane 4) obviously immunoprecipitated the recombinant protein, but the longitudinal sera of 1 patient with SLE were only weakly positive in the IPP (Fig. 3, lanes 5–7). Besides, all 9 sera whose ELISA titers were from mean + 3SD to mean + 5SD were negative in IPP (data not shown).

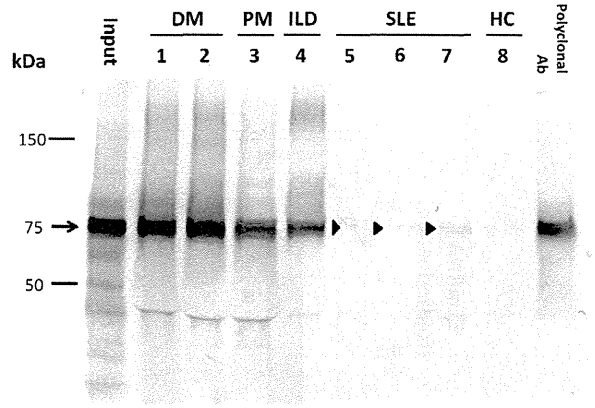

Fig. 3. Immunoprecipitation using biotinylated recombinant EJ protein. Input is half of the dose (2 μl) of biotinylated EJ protein that was used for the immunoprecipitation. 1: patient #1 (Table 2); 2: patient #2; 3: patient number #4; 4: patient #3; 5–7: 3 longitudinal sera from patient #5; 8: healthy control serum, polyclonal Ab, mouse polyclonal anti-human EJ antibody.

Table 2
Clinical features and ELISA units of anti-EJ-positive patients.

Patient no.	Age in years	Gender M/F	Diagnosis	Gottron's sign	Heliotrope rash	ILD	Internal malignancy	ELISA units	Other antibodies
1	52	M	DM	+	+	−	−	176	
2	47	F	DM	+	−	+	−	344	
3	55	F	ILD[a]	−	−	+	−	384	SS-A
4	62	F	PM	−	−	+	−	176	
5	36	F	SLE	−	−	−	−	125	dsDNA, SS-A

[a] Related to Sjögren's syndrome. SS-A: anti-SS-A antibody; dsDNA: anti-double-strand DNA antibody.

3.3. Clinical and laboratory data for anti-EJ-positive patients

The clinical and laboratory data of the anti-EJ-positive patients are summarized in Table 2. Three patients were complicated with ILD. The clinical manifestations of ILD for these 3 patients were improved by oral prednisolone therapy, and their ILD did not have a fatal outcome. Additionally, the complication of internal malignancy was not recognized in all patients 3 years before or after the disease onset. Interestingly, the 3 patients with ILD showed the highest ELISA units, and the 2 of them had very high titers of the anti-EJ: over 300 units. The patient with ILD complicated with SS also had anti-SS-A antibody. The patient with SLE had both anti-dsDNA and anti-SS-A antibodies.

3.4. Changes in expression levels of the EJ mRNA and protein by stimulation of cytokines

We investigated the effect of the 3 cytokines—IL-1β, IFN-γ and IFN α-2a—on the expression level of the EJ mRNA and protein of the human fetal lung fibroblasts (WI-38). The cultured cells were harvested separately after stimulation with cytokines according to the time course. First, the mRNA levels of the cells were analyzed by RT-PCR. A small increasing tendency for the EJ mRNA levels was recognized in the group harvested at 24 and 72 h stimulated by IFN-α (Fig. 4). However, no significant differences in EJ mRNA levels between the cytokine-stimulated group and the untreated group were recognized in the IL-1β and IFN-γ stimulation experiments.

Next, the differences in expression levels of the EJ protein between the cytokine-stimulated group and the untreated group were investigated (Fig. 5). In this study, we investigated only the cytosolic fraction, because it is generally recognized that almost all ARS proteins exist in cytosolic fraction [8]. However, we detected no significant differences in expression levels of the EJ protein between the cytokine-stimulated group and the untreated group.

4. Discussion

There are mainly 2 methods for detecting anti-ARS antibodies. One uses immunoprecipitation of the RNA and protein targeted by autoantibodies [9]. The other uses an immunoblot kit that detects limited kinds of anti-ARS antibodies by serving as a line blot kit [23]. The present report is the first to establish an ELISA assay to detect the anti-EJ antibody. In the gold standard method of using immunoprecipitation to detect the anti-ARS antibodies, it is necessary to check both the RNA and the protein for the verification of accuracy. Our ELISA assay achieves specificity for the EJ antigen by using recombinant protein, which makes it possible to test multiple samples in a short time without using radioactive materials. There are few reports that investigate MSA by using commercial solid phase assay kits [24,25], except for anti-Jo-1 antibodies. Stone et al. reported that anti-Jo-1 antibody levels of ELISA correlate with

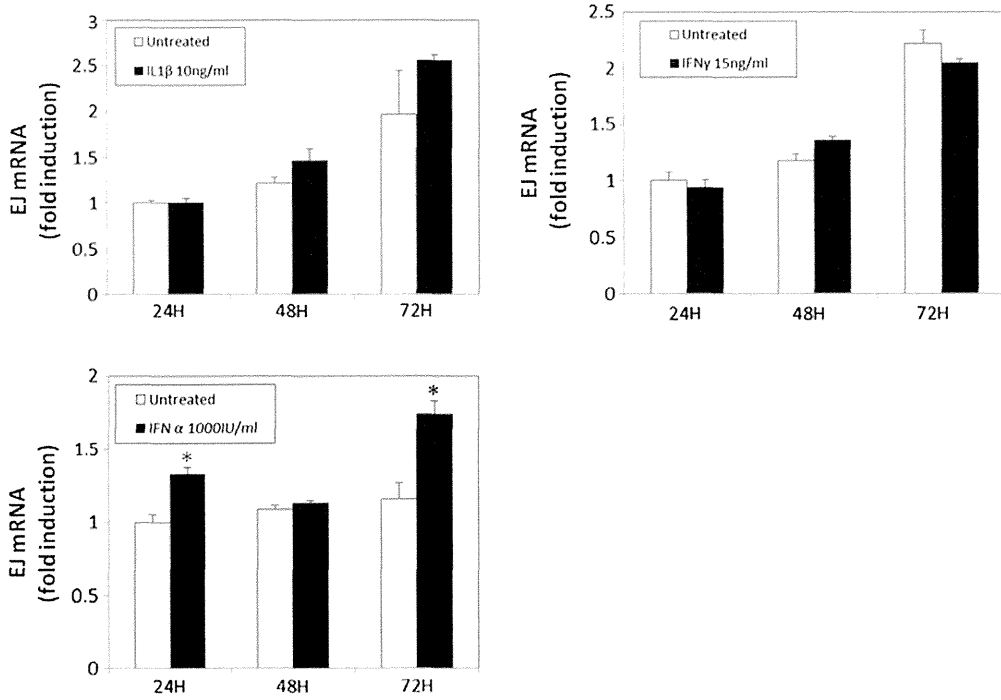

Fig. 4. RT-PCR to investigate changes in the expression level of EJ mRNA by stimulation of cytokines. After stimulation with each cytokine (IL-1β, IFN-γ, IFN-α-2a), the human fetal lung fibroblast cells (WI-38) were harvested according to the time course and levels of EJ mRNA expression were evaluated by RT-PCR analysis. GAPDH was used to normalize the gene expression. *P < 0.01. Statistical analysis between the cytokine-stimulated group and the untreated group was performed by Student's *t*-test. Error bars represent the standard deviations.

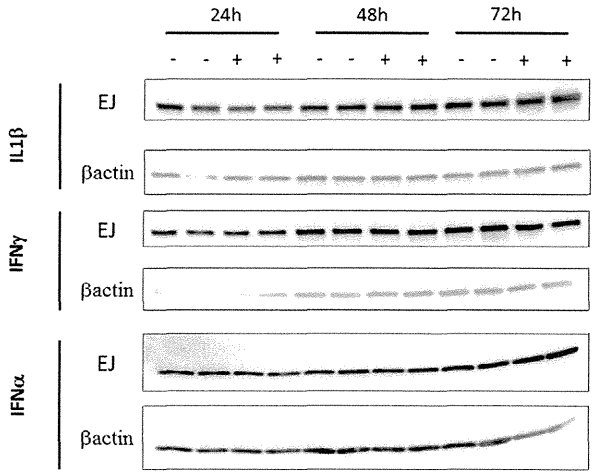

Fig. 5. Western blot to investigate changes in the expression levels of EJ protein by stimulation of cytokines. Cytokine stimulation experiments on fibroblast cells were performed same as RT-PCR experiments. EJ protein expression levels were evaluated by Western blot analysis. β-actin was used as the internal loading control of the cell lysate protein. Significant differences in the expression level of the EJ protein were not detected between the cytokine-stimulated group and the untreated group.

IIM disease activity [26]. So, establishing the ELISA assay for anti-EJ antibodies will be helpful towards understanding anti-ARS syndrome in more detail.

In this study, we screened sera from 453 patients using ELISA and found 5 anti-EJ antibody-positive patients: 2 with DM, 1 with PM, 1 with SS-ILD and 1 with SLE. In our previous study [6], sera from the 2 patients with DM had been confirmed by radio-immunoprecipitation to have anti-EJ antibodies (data not shown), although the other 3 patients' sera were not examined by such a standard method for the detection of anti-ARS. Previous studies reported that anti-EJ antibodies were recognized in 2–5% of all IIM patients [27,28]. The frequencies of anti-EJ antibody in total DM (2.3%) and in classical DM (3%) in this study are consistent with previous reports. The frequency in PM (10%) is slightly higher, probably due to the small numbers of examined PM patients. In this study, sera from the patient with SLE were obviously positive in ELISA, but only weakly positive in IPP. With regard to the discrepancy of these results, there is the possibility that the epitope of the anti-EJ of this patient may be different from those of the other patients. This patient's autoimmune epitope of the EJ protein might be cryptic and more easily bound to the autoantibody in ELISA, which is performed in more denatured condition than in IPP. Another possibility could be that the modification of the recombinant protein by the addition of spacer arm-biotin used in IPP might influence the antigenicity.

Table 3
Comparison of clinical data of patients with anti-EJ antibody of the previous report and the present study.

	Hamaguchi et al.	Present study
Age (y) at onset, mean (range)	53 (18–78)	50 (36–62)
Number (male/female)	6/32	1/4
Clinical features (at initial visit)		
ILD	84%	40%
Muscle weakness	39%	60%
Clinical features (entire follow-up period)		
ILD	97%	60%
Muscle weakness	55%	60%
Internal malignancy	3%	0%
Sjögren's syndrome	16%	40%
Heliotrope rash	21%	20%
Gottron's sign (hand)	45%	40%

The complication of ILD was recognized in 3 of the 5 anti-EJ antibody-positive patients. These 3 patients had the highest titers in ELISA. ILD is a life-threatening complication of anti-ARS syndrome [2]. Further studies using longitudinal sera from ILD patients with anti-EJ are needed to determine whether it is useful to measure anti-EJ ELISA titers for the assessment of ILD and therapeutic strategies.

We compared the clinical features of anti-EJ-positive patients reported in the multicenter study by Hamaguchi et al. [9] to those in our present study (Table 3). The age distributions and sex ratios were similar. The most common symptom was ILD, followed by muscle weakness in the entire follow-up period in both studies. Although we experienced no patient affected with malignancy, Hamaguchi et al. reported 1 patient complicated with malignancy (nasopharyngeal cancer) with anti-EJ. In their report, CADM patients accounted for 18% of the anti-EJ antibody-positive patients, whereas there was no CADM patient in our study. Unfortunately, since they did not mention the total numbers of CADM patients examined, we cannot compare frequencies of anti-EJ-positive patients in CADM. Since 1 SSc patient and 1 SLE patient were found in their and our study, respectively, anti-EJ is not always specific to DM/PM and ILD.

Many studies have mentioned that cytokines, TNF family members, interleukins, and type I and II interferons are associated with the pathogenesis of IIMs [29–32]. For example, Somani et al. reported a case in which interferon-β treatment of a multiple sclerosis patient induced severe DM [33]. Another case was reported of a patient who had received PEG-interferon α-2b therapy and then developed DM [34]. We investigated whether stimulation by type I and II interferon and IL-1β of human normal lung fibroblasts influences the expression level of the EJ mRNA and protein. We hypothesized that the change of expression level of EJ protein affects the pathogenesis of ILD. Casciola-Rosen et al. demonstrated Jo-1 protein overexpression in the DM patient's muscle tissues immunohistochemically [35]. Mammen et al. demonstrated that, in a mouse model, expression of Mi-2 protein, one of the autoantigens recognized by MSAs, in muscle tissues increases after toxic drug injection [36]. We hypothesized that the EJ protein overexpression in lung would be induced by certain cytokines and that this phenomenon might affect the pathogenesis of IIM and ILD. However, in this study, we were unable to demonstrate the increased expression of EJ protein in the normal lung fibroblasts by stimulation by any of the 3 cytokines. In previous studies, Jo-1 and Mi-2 protein overexpressions were demonstrated in the injured condition of muscle cells. The regenerating condition after injury might be important in the changes of the autoantigen's expression levels by the stimulation of cytokines.

In this study, mRNA of EJ increased only by IFN α-2a stimulation. This phenomenon may indicate the possibility that the condition of the fibroblasts started to shift to increase EJ protein production and inflammation. Recently, many researchers reported that micro RNAs (miRNAs) influence the pathogenesis of autoimmunity [37,38]. miRNAs are small, non-coding RNAs that regulate the expression of various genes at the post-transcriptional stage by inhibiting mRNA translation and degeneration [39,40]. Once the condition of the cells starts to shift to an increase in EJ-protein production by IFN-α stimulation, normal miRNAs in the normal fibroblasts (WI-38) may regulate the excess inflammation and translation of EJ-mRNA, which might finally counteract the increase in EJ-protein production.

5. Conclusion

The detection of anti-ARS antibodies including anti-EJ has been possible only at the few institutions that are able to perform radioimmunoprecipitation. We developed an ELISA to detect anti-EJ antibody by using recombinant EJ protein. It was found that anti-EJ antibodies are not specific to DM/PM and are present in other autoimmune conditions. The frequent presence of ILD in patients with high titers of anti-EJ suggests a close association of that antibody with ILD. The present newly established method for measuring anti-EJ should be carefully evaluated

in large international cohorts of patients with IIM and other autoimmune conditions, as well as ILD, toward establishing its utility and clinical significance.

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SHORT COMMUNICATION

Annular Erythema Associated with Sjögren's Syndrome Preceding Overlap Syndrome of Rheumatoid Arthritis and Polymyositis with Anti-PL-12 Autoantibodies

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Anti-PL-12 are among the anti-aminoacyl tRNA synthetase autoantibodies (1). Anti-PL-12 has been recently found in overlap syndrome of rheumatoid arthritis (RA) and polymyositis (PM)/dermatomyositis (DM), as well as in antisynthetase syndrome (2, 3). However, no skin manifestations preceding overlap syndrome with anti-PL-12 have been described.

CASE REPORT

A 44-year-old woman exhibited swelling with erythema on the bilateral upper eyelids. She was treated with prednisolone 20 mg/day at a different hospital. However, after the prednisolone was discontinued, the lesions relapsed. Four months after onset, the patient came to our clinic. A lip biopsy to investigate possible Sjögren's syndrome (SS) confirmed lymphocytic infiltration of the minor salivary glands. Circulating autoantibodies to SS-A (anti-SS-A) were positive, but anti-SS-B antibodies were negative. She had no muscle weakness at any site of the body. Serum levels of creatinine kinase were not elevated. She was diagnosed with SS according to the Japanese Ministry of Health revised criteria for the diagnosis of SS (4). The lesions resolved with oral prednisolone 30 mg/day.

When the prednisolone dose was reduced to 22.5 mg/day, several lesions of annular erythema (AE) appeared on the lower back (Fig. 1A). A biopsy of the AE lesion showed sleeve-like perivascular and periappendigeal lymphocytic infiltration, which was consistent with AE with SS (AESS) (Fig. 1B, C). The lesions resolved with oral prednisolone 30 mg/day and azathioprine 50 mg/day.

Sixteen months after the onset of SS, arthralgia on the bilateral hip, knee, and ankle joints occurred. Oral prednisolone had been tapered to 10 mg/day, and oral azathioprine 50 mg/day had been maintained. Physical examinations found swelling of the bilateral knee and ankle joints. Circulating anti-CCP antibodies were 453 U/ml (normal value < 15 U/ml). Serum levels of c-reactive protein and matrix metalloproteinase-3

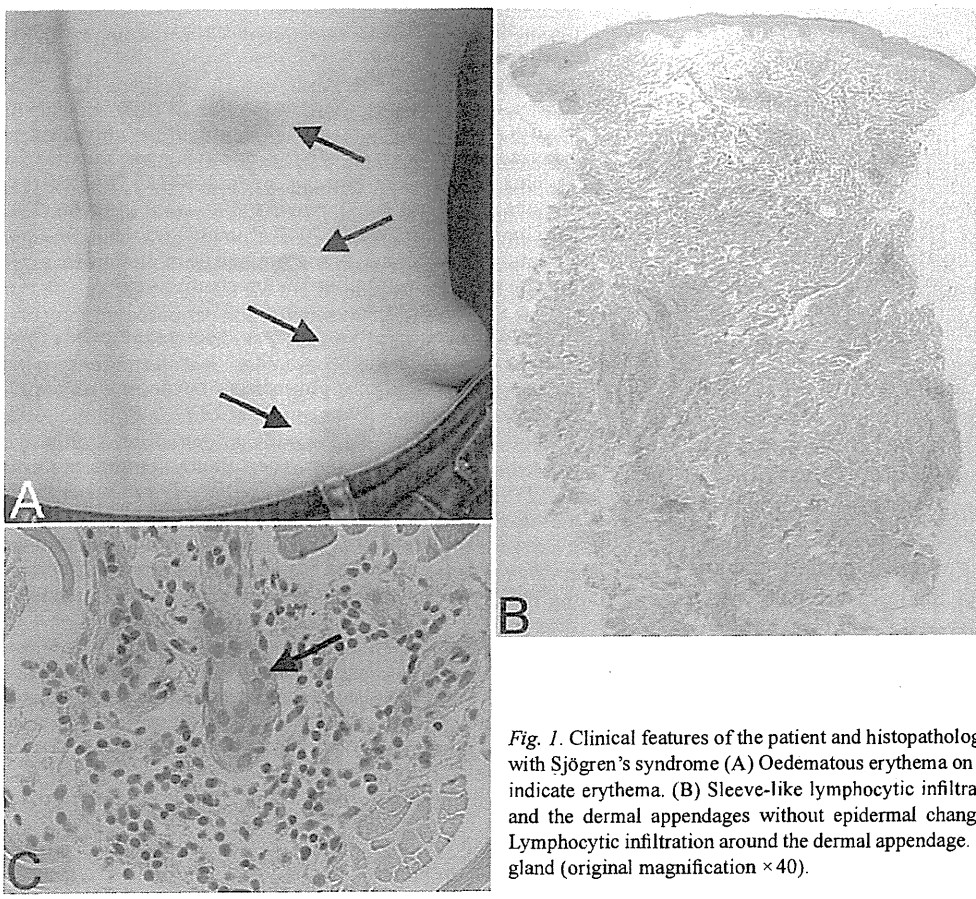

Fig. 1. Clinical features of the patient and histopathological features of the annular erythema with Sjögren's syndrome (A) Oedematous erythema on the lower back of the patient. Arrows indicate erythema. (B) Sleeve-like lymphocytic infiltrates are prominent around the vessels and the dermal appendages without epidermal changes (Original magnification ×2). (C) Lymphocytic infiltration around the dermal appendage. The arrow indicates the eccrine sweat gland (original magnification ×40).

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were elevated. The arthritis continued for >6 weeks. From these clinical features, she was diagnosed with RA according to the 2010 American College of Rheumatology/European League Against Rheumatism classification criteria for RA (5). DAS28-ESR was 4.83 (moderate activity). In addition to oral prednisolone 7.5 mg/day and azathioprine 50 mg/day, salazosulfapyridine (SASP) 500 mg/day was started and, 7 months later, methotrexate 6 mg/week was substituted for the SASP. Thereafter, the RA disease activity was controlled. azathioprine was administered for a total of 28 months.

Thirty-one months after the onset of SS, she had symptoms of refractory cough and muscle weakness in the proximal limbs. She was admitted to our hospital. The serum level of myoglobin was 546 ng/ml (normal value <60 ng/ml), creatinine kinase was 683 IU/l (<163 IU/l), aldolase was 18.5 IU/l (<5.9 IU/l), and KL-6 was 917 U/ml (<500 U/ml). Interstitial lung disease (ILD) was identified by computer tomography. Myositis in the proximal limbs was suggested by magnetic resonance imaging. Circulating anti-PL-12 was positive by ELISA, using a system developed by us. The titre of anti-PL-12 of the patient was 830 units (normal value <4 units). She was diagnosed as having PM with ILD and was treated with systemic prednisolone 50 mg/day and tacrolimus 4 mg/day. The Bohan & Peter 1975 PM/DM Criteria was used for diagnosis of PM (6, 7). PM and ILD were improved. The patient had difficulty in raising her arms and going up stairs when the creatinine kinase was 683 IU/l. After the creatinine kinase fell within the normal range, she was gradually able to extend her arms and go up the stairs. The patient has been treated with prednisolone 10 mg/day and tacrolimus 3 mg/day. We have carefully tapered prednisolone and tacrolimus, because patients with anti-PL-12 sometimes experience worsening ILD and develop pulmonary hypertension (8). prednisolone was administered for a total of 51 months.

DISCUSSION

Three clinical types of AESS have been characterised: isolated doughnut-ring-like erythema mimicking Sweet's disease with an elevated border (type I), SCLE-like marginally scaled polycyclic erythema (type II), and papular insect bite-like erythema (type III) (9). The patient had type III. According to a review of 120 cases with AESS by Katayama et al. (9), AESS can be controlled in most patients with prednisolone 5–15 mg/day, but among the patients receiving >20 mg/day of prednisolone there is a minor subset of recurrent AESS patients. The present case is categorised as the latter subset of AESS, because AESS occurred while prednisolone 22.5 mg/day was administered.

Bernacchi et al. (10) recently reported a patient with primary SS positive for anti-SS-A and anti-SS-B, who developed chronic relapsing PM and subacute cutaneous lupus erythematosus (SCLE). The present case and the previously reported case suggest a common spectrum of annular lesions of AESS and SCLE that can occur in patient with PM. When we reviewed the clinical course of the present patient, the differential diagnosis of skin manifestations of DM at the first visit could have been considered for the swelling with erythema on the bilateral upper eyelids.

In conclusion, this is the first report of annular autoimmune lesions of SS with PM and also with anti-PL-12. When we see a patient whose AESS requires >20 mg/day of prednisolone to manage, the differential diagnosis of overlap syndrome of RA and PM with anti-PL-12 should be considered.

ACKNOWLEDGEMENT

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The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Prevalence and incidence of polymyositis and dermatomyositis in Japan

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Abstract

Objectives. To estimate the number of patients with polymyositis/dermatomyositis (PM/DM) in Japan and the prevalence rate and incidence rate of the disease.
Methods. The electronic database in the nationwide registration system on intractable diseases from 2003 to 2010 was utilized to identify the number of prevalent and incident cases of PM/DM. The electronic data entry rate was used to establish the total number of registered cases.
Results. The estimated total number of patients with PM/DM and the prevalence rate in Japan in 2010 were 17,000 and 13.2 per 100,000 population, respectively. The prevalence of PM/DM ranged from 10 to 13 per 100,000 population with a trend toward increasing over time. The incidence of PM/DM was estimated within the range 10–13 per 1,000,000 person-years, except for 2003.
Conclusions. We report the prevalence and incidence of PM/DM recently in Japan for the first time at the nationwide population level. Because the prevalence seems to be increasing recently, continued monitoring of these epidemiologic features is required.

Keywords

Epidemiology, Incidence, Nationwide survey, Polymyositis/dermatomyositis (PM/DM), Prevalence

History

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Introduction

Polymyositis (PM) and dermatomyositis (DM) are chronic idiopathic inflammatory disorders, affecting the skeletal muscles, the skin and other organs. They are rare, but their chronic intractable nature has a significant impact on the utilization of medical care resources, the patients' activities of daily living, and their quality of life. The epidemiologic features of PM/DM, such as prevalence and incidence, are not well documented. In order to understand the clinical and public health importance and to plan for disease control and prevention, it is essential to estimate incidence and prevalence rates, and to know the total number affected in the population.

As PM/DM are rare diseases, only limited epidemiologic studies have been undertaken, and mostly the incidence investigated in Western countries [1–9]. Few studies have been conducted in Asian populations [10, 11]. Prevalence data on PM/DM are even more scarce [4, 11–13]. PM/DM incidence and prevalence reported in the literature have been estimated only in relatively small populations, and are likely to have correspondingly large variance. For rare diseases, epidemiological observations in large populations are required for accuracy. The incidence of PM/DM in Japan has not been estimated to date in any nationwide survey. The prevalence of PM/DM in Japan was estimated from a nationwide survey in 1991 [13]. However, no other reports on prevalence in Japan have appeared since. It is therefore worthwhile to estimate the recent incidence and prevalence of PM/DM in Japan at the nationwide population level.

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The National Program on Rare and Intractable Diseases was launched by the government in Japan in 1972 to promote research on a number of rare and intractable diseases [14]. It increased support for patients by subsidizing their health care expenditure and provides a nationwide registration system for diseases including PM/DM, systemic lupus erythematous, systemic sclerosis (SSc) and some other autoimmune diseases. In the present study, we used this database to estimate the total numbers of patients with PM/DM in Japan, and the prevalence and incidence rates of the disease.

Materials and methods

Data sources

The database in a nationwide registration system established by the Japanese government for patients with intractable diseases including PM/DM was utilized for estimating prevalence and incidence. Patients with PM/DM desiring a subsidy for their medical care, must apply for aid. If the application is accepted, the recipient is recorded in the registry. The duration of the subsidy is 1 year, effective from October to September of the following year. Renewed application and re-registration in the system are required every year, if the patients wish to continue to receive financial support. The details of the registration system have been described elsewhere [14]. Diagnoses of patients with PM/DM were established according to the standard criteria ordained by the Ministry of Health, Labour and Welfare (MHLW) of Japan [15]. Approximately 40–80% of registered patients' data were converted into electronic form, which we utilized to collect the characteristics of each patient. This electronic database has been available for epidemiological research since 2003.With permission from the MHLW, we used the data from Japanese fiscal years 2003 to 2010.

The fiscal year means from April to March of the following year. The electronic converted data indicate the year of disease onset for each patient.

Statistical analysis

We calculated the electronic data entry rate as the number of patients whose data were converted into electronic form divided by the total number of patients enrolled in the registration system for each fiscal year from 2003 to 2010. The latter information is reported in MHLW's Report on Public Health Administration and Services [16]. As the number of registered patients with PM/DM is reported together with SSc in the report, we added the number of patients with PM/DM and SSc together in the electronic converted data to give the electronic data entry rate. Thus, the electronic data entry rate is the number of PM/DM and SSc patients whose data were converted into electronic form divided by the number of all registered patients with PM/DM and SSc. Because we could not extract the electronic data entry rate exclusively for PM/DM, we assume that the data entry rate of PM/DM is not different from that of PM/DM and SSc combined. We also assume that both the entry rate of new recipients and annual renewals are the same, and also that they are identical with the rate of all patients (new and renewals summed together) in each year.

The number of PM/DM cases in each year to estimate prevalence was the number of PM/DM patients whose data were converted into electronic form divided by the electronic data entry rate.

The number of PM/DM cases to calculate incidence was estimated as follows: for the number each year, we used the onset year of the electronic converted patients. The number of patients initially registered and converted into electronic form in fiscal year-j and onset at year-i, Nij, divided by the electronic data entry rate in fiscal year-j are summed for each year-i to yield the number of incidence cases in year-i as the following equation shows:

$$Ai = \sum_{j=i-1}^{2010} \frac{Nij}{Pj}$$

i = onset year (i = 2003, 2004,..., 2010)

j = initially registered fiscal year (j = 2003, 2004,..., 2010)

$i \leq j$

Ai: number of incidence cases in year-i

Nij: number of patients initially registered and converted into electronic form in fiscal year-j and onset at year-i

Pj: electronic data entry rate in fiscal year-j

The Japanese census population in 2005 and 2010 and the estimated population in the other years are used as the denominator of the prevalence rate and incidence rate. All statistical analyses were performed with SAS version 9.1.3 software (SAS Institute Inc., Cary, NC, USA).

Ethical considerations

All data provided by the MHLW are anonymous, and researchers cannot access personal information about any of the patients.

Results

Table 1 shows the electronic data entry rate of PM/DM and SSc, and estimated number of patients with PM/DM in fiscal years 2003–2010. The number of patients with PM/DM and SSc whose data were converted into electronic form ranged from 16,388 to 33,309 each year, with the highest number in 2009. Dividing by the total number of registered patients with PM/DM and SSc enrolled in the registration system which was obtained from the MHLW's Report on Public Health Administration and Services, the electronic data entry rates were obtained. They ranged from 39% to 80%, highest in 2009. The number of registered patients with PM/DM whose data were converted into electronic form ranged from 6,328 to 13,710 which was divided by the entry rate to yield the prevalent number of patients each year. The estimated number of PM/DM patients in 2010 was 17,000, ranging in each year from 2003 to 2010 from 13,000 to 17,000, thus tending to increase over the years. These numbers can be considered to represent the total number of registered patients in the whole of Japan (population 127 million). The estimated prevalence of PM/DM in Japan in 2010 was thus 13.2 per 100,000 population. The prevalence of PM/DM ranged from 10 to 13 per 100,000 population over the years 2003–2010, increasing over time. Thus, prevalence increased 1.3-fold during this time.

The incidence of PM/DM in each year from 2003 to 2010 was estimated from the number of cases initially registered in that year, and converted into electronic form. The figures in Table 2 show the number of patients initially registered and electronically converted, excluding the cases with disease prior to 2003. The number of cases in each year is separated according to their year of onset and divided by the electronic data entry rate of that registration year to yield the estimated number of incidence cases in each year from 2003 to 2010. This ranged from 900 to 1,700 per year (Table 3). The incidence rate of PM/DM in Japan was estimated as ranging from 10 to 13 per 1,000,000 person-years (except for

Table 1. Electronic data entry rate for PM/DM and SSc (on June 2011), and estimated prevalence of PM/DM in fiscal years 2003–2010.

	PM/DM and SSc			Estimation of prevalence of PM/DM		
Fiscal year	No. of electronic entries of PM/DM and SSc patients[a]	Total No. of registered patients with PM/DM and SSc[b]	Electronic data entry rate[c]	No. of electronic entries of PM/DM patients[d]	Estimated number of patients with PM/DM[e]	Estimated prevalence (per 100,000 population)[f]
2003	20,162	31,829	0.633	8,332	13,163	10.3
2004	21,709	32,944	0.659	9,043	13,722	10.7
2005	22,057	34,592	0.638	9,327	14,619	11.4
2006	20,031	36,110	0.555	8,139	14,665	11.5
2007	16,388	37,975	0.432	6,328	14,648	11.5
2008	20,242	39,970	0.506	7,919	15,650	12.3
2009	33,309	41,648	0.800	13,710	17,138	13.4
2010	16,528	42,233	0.391	6,618	16,926	13.2

PM/DM, polymyositis/dermatomyositis; SSc, systemic sclerosis.

[a]The number of patients with PM/DM and SSc whose data were converted into electronic form.

[b]Total numbers of registered patients with PM/DM and SSc enrolled in the registration system, obtained from the Report on Public Health Administration and Services [16].

[c]a/b.

[d]The number of patients with PM/DM whose data were converted into electronic form.

[e]d/c.

[f]The Japanese census population in 2005 and 2010 and estimated population in the other years are used as the denominator of the prevalence rate.

Table 2. Number of initially registered patients with PM/DM whose data were converted into electronic form and whose year of onset year was 2003–2010.

Initially registered fiscal year	No. of patients
2003	338
2004	668
2005	921
2006	683
2007	715
2008	956
2009	1,240
2010	861
Total	6,382

PM/DM, polymyositis/dermatomyositis.

2003). The incidence rates in 2009 and 2010 are lower than in the other years.

Discussion

In this study, we estimated the number of patients with PM/DM in Japan, and also calculated prevalence and incidence of the disease based on the nationwide survey. We estimated the number of patients currently affected (e.g. prevalent cases) in Japan and its prevalence per 100,000 population. We also estimated the incidence of the disease in Japan at the nationwide population level for the first time. These results provide basic information for disease control and prevention and planning public health policy.

PM/DM are rare diseases and earlier reports on incidence and prevalence are limited. The reported incidence of PM/DM ranges from 2 to 10 per 1,000,000 person-years in different populations between the 1940s and the 1990s [1–11]. Furthermore, there is a trend toward increasing incidence over time also in these studies. Earlier prevalence data are also very limited. Estimates of prevalence from the USA [4] and Japan [11–13] range between 2.4 and 9.9 per 100,000 population. Different diagnostic and classification criteria were employed in these studies, partly explaining the diverse reported incidence and prevalence rates in these studies. Some reports determined incidence from retrospective hospital-based studies in which the true incidence of PM/DM may have been underestimated. Furthermore, as the number of patients included in most of these earlier studies was small, only 40–100, the estimates may be relatively unreliable. The increasing incidence over time may be due to an increased physician awareness of the disease, progress in diagnostic techniques or increased availability of tests and better medical records, but could also reflect a true increase in disease occurrence.

The estimated prevalence per 100,000 population of PM/DM in Japan was 10–13 in 2003–2010, tending to increase over the years. The incidence of PM/DM per 1,000,000 person-years was estimated as 10–13, except in 2003. The prevalence and incidence of PM/DM in our study are higher than in most previously reported estimates. These differences may be due to the lack of standardization of the diagnostic criteria employed, as well as different ethnicities of the patients. Easier access to medical care via the public insurance system and financial support program in Japan may be one more reason for the higher estimates in this country. Cases in this study were identified by the standard diagnostic criteria ordained by the MHLW of Japan [15]. The significance of our study depends on the clearly defined standard criteria and large scale population review. The estimated incidence is based on a large number of approximately 6,400 patients during 8 years (Table 2).

A comparable total number of PM/DM patients in Japan are also available from a nationwide survey which accumulated number of patients reported from random-sampled hospitals from all over Japan, although the methodology of that survey was different from our own. The total number of patients with PM/DM in Japan was estimated at 3,000 (95% confidence interval 2,800–3,300) for PM and the same number (95% confidence interval 2,800–3,200) for DM, in 1991 [13]. Our estimate for PM/DM in Japan was 17,000 in 2010. This implies an increase of 2.8-fold (17,000/6,000) between 1991 and 2010. Our study also determined an increase in PM/DM prevalence over time. In addition to the above-mentioned factors which manifest as apparent increases in incidence rate, such as the use of different diagnostic criteria and physician awareness of the disease, better prognosis as result of improved treatment might also account for this trend.

Our estimated incidence values in 2009 and 2010 are slightly lower than 2008. This could be an underestimate because cases which would be registered from 2011 onward were not included.

There are some limitations to this study. First, there may be a possible bias due to the use of data from the Japanese government's registration system. Regarding the accuracy of the data we used, some degree of over- and/or underdiagnosis may exist, although specialist committees organized in each prefectural government check the diagnoses according to the standard criteria ordained by the government. With respect to coverage of the patients, most are expected to be diagnosed and registered in this system, but clearly we cannot be sure of the number of omissions. Patients who do not need financial support, some pediatric patients and patients whose medical expenses are covered by local government in an alternative financial support system will not apply to the system we used and thus be missing from the database. Thus, our study may underestimate prevalence. Second, we could not distinguish between PM and DM prevalence and incidence separately. This is because the national registration system combines these two diseases for administrative reasons, even though they are subgrouped

Table 3. Estimated number of incidence cases and incidence rate of PM/DM by onset year.

	Initially registered fiscal year									
Onset year	2003	2004	2005	2006	2007	2008	2009	2010	Total[a]	Incidence per 1,000,000 person-years[b]
2003	466	209	69	40	23	32	23	3	865	6.8
2004	68	750	260	63	60	26	21	15	1,263	9.9
2005		55	1,016	204	63	61	25	49	1,473	11.5
2006			99	886	301	99	46	46	1,477	11.6
2007				38	1,146	356	78	61	1,679	13.1
2008					63	1,253	290	110	1,716	13.4
2009						63	1,026	442	1,531	12.0
2010							41	1,476	1,517	11.8

PM/DM, polymyositis/dermatomyositis.

[a]Estimated number of incidence cases.

[b]The Japanese census population in 2005 and 2010 and estimated population in the other years are used as the denominator of the incidence rate.