Table 2 Rheumatoid arthritis-susceptible and arthritis-protective *HLA* alleles[a]

	Anti-CCP positive ($n = 488$ individuals)				Anti-CCP negative ($n = 103$ individuals)				Control ($n = 1037$ individuals)
HLA allele	Freq. (%)	*P*-value	OR	95% CI	Freq. (%)	*P*-value	OR	95% CI	Freq. (%)
*DRB1*04:01*	3.1	3.35e-04	2.71	1.52–4.87	1.9	0.312	1.69	0.42–4.99	1.2
*DRB1*04:05*	29.2	1.74e-25	2.73	2.26–3.31	16.0	0.239	1.26	0.83–1.89	13.1
*DRB1*13:02*	2.3	1.03e-10	0.27	0.16–0.43	5.3	0.268	0.67	0.32–1.25	7.8
*DRB1*14:05*	0.5	4.42e-05	0.20	0.06–0.49	3.4	0.489	1.34	0.51–3.01	2.6
*DRB1*08:02*	1.4	2.56e-04	0.37	0.19–0.66	2.9	0.699	0.76	0.27–1.75	3.8
*DRB1*10:01*	1.7	1.58e-05	7.33	2.59–25.5	0.5	0.434	2.02	0.04–18.1	0.2
*DQB1*04:01*	28.5	1.46e-24	2.71	2.23–3.29	15.5	0.278	1.25	0.81–1.88	12.8
*DQB1*06:04*	2.2	9.49e-11	0.26	0.16–0.42	4.9	0.205	0.62	0.29–1.20	7.6
*DPB1*02:01*	28.5	1.99e-04	1.40	1.17–1.67	24.8	0.430	1.15	0.81–1.62	22.2
*DPB1*04:01*	2.5	9.18e-04	0.48	0.29–0.76	5.3	0.867	1.07	0.51–2.03	5.0
*DPB1*09:01*	6.4	1.85e-03	0.63	0.46–0.85	8.7	0.712	0.89	0.50–1.48	9.7
*B*15:18*	3.7	2.35e-05	3.02	1.76–5.23	1.9	0.342	1.56	0.39–4.56	1.3
*B*37:01*	1.7	1.02e-04	5.23	2.05–14.8	1.0	0.192	2.89	0.29–15.3	0.3
*B*44:03*	3.3	5.23e-09	0.35	0.23–0.52	5.8	0.152	0.64	0.32–1.17	8.8
*B*54:01*	10.2	3.40e-04	1.65	1.25–2.18	8.7	0.239	1.39	0.78–2.34	6.5
*B*59:01*	4.8	1.35e-06	3.03	1.90–4.90	2.4	0.393	1.49	0.45–3.90	1.6
*C*01:02*	22.8	1.87e-06	1.60	1.31–1.94	22.8	0.010	1.60	1.10–2.28	15.6
*C*06:02*	1.7	1.85e-03	3.32	1.46–7.88	1.0	0.331	1.84	0.20–8.51	0.5
*C*07:04*	2.6	2.50e-05	4.17	2.04–8.91	1.0	0.639	1.55	0.17–6.93	0.6
*C*14:03*	3.5	1.20e-08	0.37	0.25–0.54	5.3	0.090	0.58	0.28–1.08	8.9
*A*33:03*	5.0	1.39e-04	0.54	0.38–0.75	7.8	0.699	0.86	0.47–1.47	8.9

CCP, cyclic citrullinated peptides; CI, confidence intervals; OR, odds ratio; RA, rheumatoid arthritis.
[a]Only *HLA* alleles that showed significant association with RA after Bonferroni correction are shown. *P*-values shown here are without Bonferroni correction.

Table 3 Six HLA-loci haplotypes, their frequencies and associations with anti-CCP Ab-positive rheumatoid arthritis

HLA loci						Freq. (%)[a]				
*A**	*C**	*B**	*DRB1**	*DQB1**	*DPB1**	Case (*n*)	Control (*n*)	*P*-value	OR	95% CI
24:02	12:02	52:01	15:02	06:01	09:01	4.7 (46)	6.6 (137)	0.041	0.70	0.48–0.99
24:02	01:02	54:01	04:05	04:01	05:01	2.6 (25)	0.8 (16)	1.40e-04	3.38	1.73–6.81
24:02	07:02	07:02	01:01	05:01	04:02	2.6 (25)	2.7 (57)	0.811	0.93	0.55–1.52
33:03	14:03	44:03	13:02	06:04	04:01	1.4 (14)	3.5 (72)	1.38e-03	0.40	0.21–0.73
24:02	12:02	52:01	15:02	06:01	02:01	1.3 (13)	0.5 (11)	0.027	2.53	1.04–6.26
24:02	12:02	52:01	15:02	06:01	05:01	1.1 (11)	0.8 (16)	0.407	1.47	0.61–3.38
26:01	03:04	40:02	09:01	03:03	05:01	1.1 (11)	0.5 (11)	0.105	2.14	0.84–5.46
02:06	01:02	59:01	04:05	04:01	04:02	0.9 (9)	0.3 (6)	0.026	3.21	1.02–10.9
24:02	01:02	59:01	04:05	04:01	04:02	0.9 (9)	0.7 (14)	0.503	1.37	0.52–3.41
24:02	01:02	59:01	04:05	04:01	05:01	0.8 (8)	0.1 (3)	6.60e-03	5.70	1.36–33.5
02:06	01:02	54:01	04:05	04:01	05:01	0.7 (7)	0.3 (6)	0.132	2.49	0.71–8.99
01:01	06:02	37:01	10:01	05:01	02:01	0.6 (6)	0.2 (5)	0.117	2.56	0.65–10.6
02:01	01:02	54:01	04:05	04:01	05:01	0.6 (6)	0.2 (4)	0.084	3.20	0.76–15.4
02:10	08:01	40:06	04:05	04:01	05:01	0.6 (6)	0.2 (4)	0.084	3.20	0.76–15.4
11:01	04:01	15:01	04:06	03:02	02:01	0.6 (6)	0.7 (15)	0.819	0.85	0.27–2.32
11:01	07:02	39:01	08:03	06:01	02:01	0.6 (6)	0.2 (5)	0.117	2.56	0.65–10.6

Ab, antibody; CCP, cyclic citrullinated peptides; CI, confidence intervals; OR, odds ratio.
[a]Only the haplotypes with more than 0.6% (more than six chromosomes) of frequency in cases are shown.

(Tables 3 and 4). Almost all of the haplotypes that were significantly increased or significantly decreased in RA patients carried RA-susceptible or RA-protective *DRB1* alleles. Therefore, the significant association of particular *HLA* alleles, other than *DRB1* alleles with RA (Table 2) could be explained by LD with DRB1 alleles in the same haplotypes. The exceptions were the DPB1 alleles: *DPB1*02:01*, *DPB1*04:01* and *DPB1*09:01*.

Table 4 Four HLA-loci haplotypes, their frequencies and associations with anti-CCP Ab-positive rheumatoid arthritis

HLA loci				Freq. (%)[a]				
*C**	*B**	*DRB1**	*DQB1**	Case (*n*)	Control (*n*)	*P*-value	OR	95% CI
12:02	52:01	15:02	06:01	8.4 (82)	9.5 (197)	0.346	0.87	0.66–1.15
01:02	54:01	04:05	04:01	7.2 (70)	3.8 (78)	8.92e-05	1.98	1.40–2.79
07:02	07:02	01:01	05:01	4.6 (45)	5.7 (118)	0.228	0.80	0.55–1.15
01:02	59:01	04:05	04:01	4.3 (42)	1.4 (30)	4.31e-06	3.06	1.86–5.10
01:02	46:01	08:03	06:01	2.4 (23)	2.7 (56)	0.627	0.87	0.51–1.45
03:04	40:02	09:01	03:03	2.4 (23)	1.6 (33)	0.149	1.49	0.83–2.64
08:01	40:06	09:01	03:03	2.4 (23)	2.2 (46)	0.795	1.06	0.61–1.80
07:04	15:18	04:01	03:01	2.0 (20)	0.4 (9)	5.81e-05	4.80	2.08–12.0
14:03	44:03	13:02	06:04	1.9 (19)	7.1 (147)	3.45e-10	0.26	0.15–0.42
01:02	55:02	04:05	04:01	1.7 (17)	0.7 (14)	0.010	2.61	1.20–5.74
06:02	37:01	10:01	05:01	1.6 (16)	0.2 (5)	3.88e-05	6.89	2.40–24.1
03:04	40:01	04:05	04:01	1.5 (15)	0.3 (7)	7.15e-04	4.60	1.76–13.4
04:01	15:01	04:06	03:02	1.5 (15)	1.8 (38)	0.657	0.836	0.43–1.57
14:02	51:01	04:05	04:01	1.5 (15)	1.2 (24)	0.391	1.33	0.65–2.66
03:04	40:02	04:05	04:01	1.3 (13)	0.2 (5)	5.28e-04	5.58	1.86–20.1
08:01	48:01	09:01	03:03	1.2 (12)	0.4 (9)	0.018	2.86	1.10–7.70
03:03	35:01	15:01	06:02	1.0 (10)	0.7 (15)	0.394	1.42	0.57–3.39
03:03	35:01	04:05	04:01	1.0 (10)	1.0 (21)	1.00	1.01	0.42–2.26

Ab, antibody; CCP, cyclic citrullinated peptides; CI, confidence intervals; OR, odds ratio.
[a]Only the haplotypes with more than 1.0% (more than 10 chromosomes) of frequency in cases are shown.

The haplotypes of *DPB1*02:01* contained *DRB1*15:02, *10:01, *04:06* and **08:03* at frequencies of 1.3%, 0.6%, 0.6% and 0.6%, respectively (Table 3). Therefore, the majority of the *DRB1* alleles in the same haplotype as *DPB1*0201* were neutral to RA susceptibility because *DRB1*15:02, *04:06,* and **08:03* were neutral to RA susceptibility. *DRB1*10:01* was the only RA-susceptible allele (Table 2).

In order to assess the independent association of *DPB1*02:01* from *DRB1*10:01*, which was RA susceptible, we performed Fisher's exact test after exclusion of *DRB1*10:01*. The association between *DPB1*02:01* and anti-CCP Ab-positive RA, after exclusion of *DRB1*10:01*, is significant even after Bonferroni correction ($P = 0.00107$, OR = 1.34, 95% CI = 1.12–1.61). Furthermore, both the *DRB1*04:05* and *DPB1*02:01* positive cases showed a higher OR than the cases with either *DRB1*04:05* or *DPB1*02:01* positivity : *DRB1*04:05/DPB1*02:01* double positive *vs* double negative ($P = 3.71e-17$, OR = 5.43, 95% CI = 3.61–8.20), *DRB1*04:05* positive *vs DRB1*04:05* negative (dominant mode, $P = 5.44e-23$, OR = 3.55, 95% CI = 2.74–4.60) and *DPB1*02:01* positive *vs DPB1*02:01* negative (dominant mode, $P = 7.28e-03$, OR = 1.40, 95% CI = 1.09–1.79).

The *DPB1*04:01* allele was in the same haplotype as *DRB1:13:02*, which was RA protective (Table 3). A similar result was obtained for the protective effect of *DPB1*04:01* after the exclusion of *DRB1*13:02* from the assessment ($P = 9.25e-10$, OR = 0.16, 95% CI = 0.07–0.34). The *DPB1*09:01* allele was RA-protective (Table 2) and the major haplotype with *DPB1*09:01* is *DRB1*15:02*, which was neutral for the onset of RA (Table 3). Furthermore, there is no other haplotype carrying *DPB1:09:01* among the haplotypes with a frequency of more than 0.6% (Table 3). These results indicate that the associations between DPB1 alleles and anti-CCP Ab-positive RA were independent from the DRB1 locus.

Association between HLA-haplotypes and anti-CCP Ab-positive RA

*DRB1*04:01* is one of the RA-susceptible alleles in both Caucasians and Japanese (10). The HLA alleles of *C*07:04, B*15:18, DRB1*04:01* and *DQB1*03:01* were found on the same haplotype (Table 4). The *P*-values and ORs for *C*07:04* and *B*15:18* were comparable to those for *DRB1*04:01* (Table 2).

There were several haplotypes carrying particular RA-sensitive or RA-protective *HLA* alleles. For example, there were eight six-HLA-loci haplotypes and seven four-HLA-loci haplotypes carrying *DRB1*04:05* among the haplotypes with more than 0.6% of frequency for the six-HLA-loci haplotypes and with more than 1.0% of frequency for the four-HLA-loci haplotypes in the cases (Tables 3 and 4). Among these haplotypes, the *P*-values and the ORs varied from 1.40e-04 to 0.503 and from 5.70 to 1.37 in the six-HLA-loci haplotypes and from 4.31e-06 to 1.00 and from 5.58 to 1.01 in the four-HLA-loci haplotypes, respectively.

Association between HLA genotypes and anti-CCP antibody-positive RA

When the association between *HLA* genotypes and anti-CCP Ab-positive RA was analyzed, only the *DRB1* and *DQB1* loci

Table 5 The Cochran–Armitage test for trends on the genotypes of *HLA* alleles[a]

HLA allele	Chi-squared value	*P*-value
*DRB1*04:01*	15.0	1.08e-04
*DRB1*04:05*	151	1.22e-34
*DRB1*13:02*	39.0	4.25e-10
*DRB1*14:05*	15.1	1.03e-04
*DQB1*04:01*	145	2.47e-33
*DQB1*06:04*	38.1	6.80e-10
*DPB1*02:01*	23.8	1.08e-06
*DPB1*04:01*	9.43	2.14e-03
*DPB1*09:01*	11.4	7.20e-04
*B*15:18*	20.9	4.87e-06
*B*44:03*	34.5	4.27e-09
*B*54:01*	12.8	3.56e-04
*B*59:01*	27.3	1.71e-07
*C*01:02*	26.9	2.15e-07
*C*14:03*	32.8	1.02e-08
C*06:02	9.00	2.71e-03
*A*33:03*	16.9	3.89e-05

[a]*DRB1*08:02*, *DRB1*10:01*, *B*37:01* and *C*07:04* were omitted because there was no homozygote for these alleles.

showed significant associations after Bonferroni correction using the number of genotypes observed in this study: 67 for *HLA-A*, 82 for *HLA-C*, 179 for *HLA-B*, 123 for *DRB1*, 62 for *DQB1* and 47 for *DPB1*. RA was associated significantly with *DRB1*04:05/04:05* (34 cases and 18 controls, $P = 5.43e\text{-}07$, OR = 4.24, 95% CI = 2.30–8.06), *DRB1*04:05/09:01* (50 cases and 42 controls, $P = 5.06e\text{-}06$, OR = 2.70, 95% CI = 1.73–4.24) and *DRB1*04:01/04:05* (14 cases and 3 controls, $P = 2.50e\text{-}05$, OR = 10.1, 95% CI = 2.82–55.4). The *DRB1*04:05* homozygote showed a higher OR than *DRB1*04:05* in the allele mode at 4.24 and 2.73, respectively.

To assess the double-dose effects of *HLA* alleles, we divided the genotypes into AA (homozygote of A alleles), Aa (heterozygote of A allele) and aa (without A allele) and found that all of the *HLA* alleles that were RA susceptible or RA protective in the assessment using the allele mode (Table 2) also showed significant *P*-values in the Cochran–Armitage test (Table 5). These results indicated that the homozygotes of RA-susceptible or RA-protective *HLA* alleles confer either a higher risk or lower risk than the heterozygotes.

Association between SE and anti-CCP antibody-positive RA

The *DRB1* alleles based on the SE classification (12) were S2 ($P = 3.35e\text{-}04$, OR = 2.71, 95% CI = 1.52–4.89) and S3p ($P = 6.55e\text{-}20$, OR = 2.14, 95% CI = 1.81–2.53) for RA-susceptible SEs, and S1 ($P = 2.14e\text{-}08$, OR = 0.58, 95% CI = 0.48–0.71) and X ($P = 1.22e\text{-}04$, OR = 0.73, 95% CI = 0.62–0.86) for RA-protective SEs. Genotypes associated with these SEs showed a significant increase or decrease in the anti-CCP Ab-positive RA patients (data not shown). The Cochran–Armitage test for trends showed the double-dose effect for S3p ($P = 6.50e\text{-}21$), S2 ($P = 2.09e\text{-}04$), S1 ($P = 3.33e\text{-}08$) and X ($P = 1.10e\text{-}04$) in the anti-CCP Ab-positive RA patients. However, some *DRB1* alleles including *DRB1*01:01* in the S3p SE group were not associated with anti-CCP Ab positive RA (Table 6). Although *DRB1*01:01* was reported to be an RA-susceptible allele in Caucasians (10, 35), we could not detect a significant association between *DRB1*01:01* and RA.

Association between disease types and HLA alleles or anti-CCP antibody positivity

We divided the RA patients into three groups, MUD, MES and LES based on their disease types (Table 1), namely on the degree of erosiveness and the time course of joint disruption according to a previous report (27). However, the MUD and MES were ultimately combined into one group (MUD/MES) for statistical analyses because they showed more serious erosive arthritis than LES and there were only 17 cases of MUD and 16 cases of anti-CCP Ab-positive MUD out of the 427 cases collected from Tokai University Hospital in this study. The prevalence of MUD in the current study was 4.0% and showed good concordance with the ~5% prevalence in a previous report (36). There were no significant differences between (MUD/MES)-susceptible *HLA* alleles and LES-susceptible *HLA* alleles by the Fisher's exact test (data not shown). SE positivity also showed no association with disease types. However, the *DPB1*02:01* frequency was higher MUD/MES ($P = 1.83e\text{-}03$, OR = 1.40, 95% CI = 1.13–1.74) than in LES ($P = 0.142$, OR = 1.25, 95% CI = 0.92–1.68). Anti-CCP Ab-positive RA was associated more significantly with MUD/MES than with LES ($P = 0.00675$, OR = 2.14, 95% CI = 1.22–3.74) as previously reported (37, 38).

Discussion

In this study the sero-negative RA was not associated with any alleles in *HLA-A*, *-B*, *-C*, *-DQB1* and *-DPB1* loci or any alleles in the *DRB1* locus. The SE in *DRB1*, which is defined by the amino acids positioned at residues 70–74, was associated with the autoantibody-positive but not sero-negative RA as previously reported (29). However, not all the *DRB1* alleles with the SE S3p, which confers RA susceptibility, were RA susceptible (Table 6). *DRB1*04:05*, **10:01* and **04:04* were RA susceptible and *DRB1*01:01*, **04:10* and **14:06* were neutral in this study, although *DRB1*01:01* was previously reported as either neutral (34) or RA susceptible (39) in the East Asian population.

Collagen-induced arthritis (CIA) and collagen II (CII) immunization in RA-susceptible allele transgenic mice has been used as an animal model of RA (40). The enhancement of T-cell responses to type II collagen (CII) and its

Table 6 Associations between *DRB1* alleles/SEs and anti-CCP Ab-positive rheumatoid arthritis[a]

DRB1 allele	SE	Number of chromosomes: Case ($n = 976$)	Number of chromosomes: Control ($n = 2074$)	*P*-value	OR	95% CI
0401	S_2	30	24	3.35e-04	2.71	1.52–4.87
0101	S_{3p}	61	142	0.586	0.907	0.65–1.25
0404	S_{3p}	5	1	0.015	10.7	1.19–504
0405	S_{3p}	285	272	1.74e-25	2.73	2.25–3.31
0410	S_{3p}	18	45	0.588	0.85	0.46–1.50
1001	S_{3p}	17	5	1.58e-05	7.33	2.59–25.5
1406	S_{3p}	13	42	0.192	0.65	0.32–1.25
1301	S_1	1	14	0.048	0.15	0.00–1.00
1302	S_1	22	162	1.03e-10	0.27	0.16–0.43
1501	S_1	50	136	0.144	0.77	0.54–1.08
1502	S_1	91	223	0.250	0.85	0.65–1.09
0403	X	18	72	0.015	0.52	0.29–0.89
0406	X	21	62	0.232	0.71	0.41–1.20
0407	X	4	8	1.00	1.06	0.23–3.98
0802	X	14	79	2.56e-4	0.37	0.19–0.66
0803	X	60	149	0.318	0.85	0.61–1.16
0901	X	166	315	0.202	1.14	0.93–1.41
1401	X	22	62	0.348	0.78	0.46–1.29
1403	X	7	29	0.149	0.51	0.19–1.19
1405	X	5	53	4.42e-05	0.20	0.06–0.49
1101	S_{3D}	10	44	0.038	0.48	0.21–0.96
1201	S_{3D}	27	77	0.200	0.74	0.45–1.17
1202	S_{3D}	15	36	0.764	0.88	0.45–1.66
1602	S_{3D}	8	13	0.639	1.31	0.47–3.42

Ab, antibody; CCP, cyclic citrullinated peptides; CI, confidence intervals; OR, odds ratio; SEs, shared epitopes.
[a]*DRB1:03:01, DRB1*07:01, DRB1*12:05, DRB1*13:07, DRB1*14:02, DRB1*14:07, DRB1*14:12, DRB1*08:09* and **08:23* were omitted because of the small number of cases and controls in this study: the number of chromosomes is less than five in both of cases and controls.

immunodominant epitope CII (aa 255–274) was observed in RA patients (41). DRB1-Arg^{71} in the SE region and accommodated to the P4 binding pocket certainly interacts with the Glu^{266} residue of human CII (aa 259–273) (42). These findings as well as those of many other studies indicate the importance of SE in the onset of RA. However, the peptide-binding motif of the HLA groves is determined not only by P4 but also by P1, P6, P7 and P9. It is likely that RA-related peptide binding to *DRB1*01:01*, **04:10* and **14:06*, which are neutral to RA susceptibility in this study, are affected by the polymorphisms in the peptide amino acids interacting with peptide-binding pocket of DR molecules.

We showed in this study that *DPB1*02:01* confers RA susceptibility, whereas *DPB1*04:01* and **09:01* confer RA protection (Table 2). These associations were independent from the *DRB1* locus. The single nucleotide polymorphism (SNP) rs3117213, which is downstream of the *DPB1* locus, was reported to be associated with RA independently of *DRB1* (43). An independent association of the *HLA-DPB1-COL11A2* locus with RA was also reported (44). More recently, it was reported that single-amino-acid polymorphism in *HLA-DPB1* (at position 9) contributes to the MHC association to RA risk (45). Our results are in agreement with this report except for *DPB1*04:01* which has RA-susceptible amino acid, phenylalanine, at position 9 (45). This difference between the studies may be because of the use of different methods with the use of amino acid polymorphism in the previous report (45) and *HLA* alleles in this study. In this context, further studies are required to clarify the relations among *DPB1*02:01* amino acid polymorphism at position 9 in DPB1 and rs3117213 in the *DPB1-COL11A2* region.

*DPB1*02:01* was also associated with anti-CCP Ab-positive RA and more erosive RA. In addition, a statistically significant increase of *DPB1*02:01* among early-onset pauciarticular juvenile RA and a positive interaction between *DPB1*0201* and the *DRB1* alleles encoding DR3, DR5 or DR6 were reported (46). This suggests that *DRB1*04:05* and *DPB1*02:01* may exert a collective effect on the onset of RA to bring about a more erosive effect in Japanese patients.

*HLA-B*15:18* and *C*07:04* showed comparable ORs and *P*-values to *DRB1*04:01*. These four HLA alleles, *C*07:04, B*15:18, DRB1*04:01* and *DQB1*03:01* are on the same haplotype (Table 4). There are some RA-susceptible genes/SNPs in the MHC class III region between DRB1 and HLA-B, which are possibly in LD with the *B*15:18-C*07:04* haplotype that may have contributed to the OR and

P-value of the *B*15:18-C*07:04* haplotype. For example, there are several RA-susceptible genes/SNPs reported in the class III region such as *NFKBIL1*, *TNF*, *TNXB*, *NOTCH4* and *AIF1* (11–19), as well as non-HLA loci in the class I region such as MICA and VARS2L (47, 48). Alternatively, our result, which showed comparable ORs and lower *P*-values of *HLA-B*15:18* and *C*07:04* to *DRB1*04:01*, may reflect the differences between populations, because in some population-based studies, the TNF region was associated with RA susceptibility independently of the HLA-DRB1 association (49). Further analyses of haplotypes focusing on HLA class I, class II and class III regions should be performed in various populations.

Some haplotypes with *DRB1*04:05*, which was the most RA-susceptible allele in the Japanese, were neutral in RA. Namely, the OR varied from 1.01 to 5.58 depending on the *HLA-B* and *HLA-C* alleles that were present in the four HLA-loci haplotypes with *DRB1*04:05* and *DQB1*04:01* (Table 4). These findings highlight the importance of examining haplotypes that include the *HLA* class I, class II and class III regions in RA. All three *HLA* regions should be elucidated, as extended haplotypes in order to better understand their contribution to the onset and maintenance of RA. Finally, our findings suggest that various *HLA* class I loci and alleles contributed to the onset of RA with or without the cooperation of the *DRB1* alleles. As genetic association with RA in Japanese RA patients might be different in Western individuals, replication studies in Western RA patients are required to confirm the results shown in the present study.

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S. M., Y. S. and H. I. designed the study, Y. S., M. K, S. S. Y. K. and Y. H. contributed samples and K. K. and Y. O. and S. M. performed the experiments. S. M., A. N. J. K. K. and I. I. analyzed the data, S. M. J. K. K and H. I. wrote the paper, J. K. K., I. I., A. N., M. K., S. S. and Y. S. read and substantially commented on the manuscript, and H. I. provided financial support.

Conflict of interest

The authors have declared no conflicting interests.

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ORIGINAL CONTRIBUTION

Myopathy Associated With Antibodies to Signal Recognition Particle

Disease Progression and Neurological Outcome

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Objective: To characterize the clinical course of myopathy associated with antibodies to signal recognition particle (SRP), or anti-SRP myopathy.

Design: Case series.

Setting: Keio University Hospitals and National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, Japan.

Patients: We reviewed clinical features of 27 patients with anti-SRP myopathy and analyzed disease progression and neurological outcome.

Main Outcome Measures: Anti-SRP antibodies in serum were detected by RNA immunoprecipitation assay using extracts of K562 cells.

Results: Of the 27 patients, 5 (19%) showed chronic progressive muscle weakness as well as atrophy of limbs and trunk muscles from a younger age with more severe neurological outcomes compared with the other 22 patients (81%) with the subacute form.

Conclusion: A subset of patients with anti-SRP myopathy can show a chronic progressive form associated with severe clinical deficits.

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AUTOANTIBODIES AGAINST signal recognition particle (SRP) were first found in the serum of a patient with polymyositis and were listed as myositis-specific antibodies.[1] Myopathy associated with antibodies to SRP (anti-SRP myopathy) has recently been regarded as an immune-mediated necrotizing myopathy based on histological findings and has been clinically characterized by severe muscle weakness, marked elevation of serum creatine kinase (CK) levels, and poor response to corticosteroid therapy.[2-7] These observations were gathered mainly from patients with a clinical diagnosis of inflammatory myopathies. However, the clinical spectrum of anti-SRP myopathy may be broader.

The rapid progression of weakness is a characteristic clinical feature of anti-SRP myopathy.[2-7] The mean interval from its onset to diagnosis is 3 to 4 months, and clinical symptoms are usually progressive for 5 to 6 months.[3-5] In contrast, Dimitri et al[8] first described a 31-year-old man in whom weakness progressed for more than 3 years. Before the anti-SRP antibody was detected, he was diagnosed as having limb-girdle muscular atrophy. We also described a 32-year-old man with childhood-onset myopathy whose diagnosis alternated between inflammatory myopathy and muscular dystrophy for 21 years.[9] These results suggested that patients with anti-SRP myopathy can show chronic progression indistinguishable from muscular dystrophy. Herein, we analyzed the disease course and neurological outcomes in patients with anti-SRP myopathy.

METHODS

We chose 27 patients with myopathy with the anti-SRP antibody, including 10 previously reported cases.[9,10] The diagnosis of anti-SRP myopathy was based on clinical, electrophysiological, histopathological, and serological findings. Muscle weakness was assessed by manual muscle strength (Medical Research Council scale grade), and severe weakness was defined as grade 3 or lower. Muscle biopsy was performed in all 27 patients and showed fiber size variation as well as fiber necrosis and regeneration with or without lymphocyte infiltration. No patients had taken statins.

Anti-SRP antibodies were detected by RNA immunoprecipitation assay using extracts of K562 cells as previously described.[11] Briefly, 10 μL of serum was mixed with 2 mg of Protein A Sepharose CL-4B (Pharmacia Biotech AB) in 500 μL of immunoprecipitation buffer (10mM TRIS hydrochloride, pH 8.0, 500mM sodium chloride, 0.1% Nonidet P40) and incubated for 2 hours. After washing 3 times with immunoprecipitation buffer, antigen-bound Sepharose beads were mixed with 100 μL of K562 cell extract (6×10^6 cell equivalents per sample) for 2 hours, and 30 μL of 3M sodium acetate, 30 μL of 10% sodium dodecyl sulfate, and 300 μL of phenol:chloroform:isoamyl alcohol (50:50:1, containing 0.1% 8-hydroxyquinoline) were added to extract bound RNA. After ethanol precipitation, the RNA was resolved by using a 7M urea–8% polyacrylamide gel, and the gel was silver stained (Bio-Rad). Immunoprecipitated RNA located in the 7SL-RNA lesion was regarded as anti-SRP antibody. Other myositis-specific and myositis-associated autoantibodies were also detected by the RNA immunoprecipitation assay.

Neurological outcomes were assessed using the modified Rankin Scale (mRS).[12] This scale was principally used for evaluating function of patients with stroke; however, it was also applied to patients with myositis.[13] Neurological outcomes were divided into 3 groups: recovered, mild deficit, and severe deficit. Patients who responded optimally to the treatment and returned to their jobs (mRS score of 0-1) were defined as recovered. Patients who responded partially to treatment and resumed most activities of daily living (mRS score of 2-3) were defined as having a mild deficit. Patients who showed reworsening muscle weakness or re-elevation of serum CK levels after the treatment were also included in this group. Patients who responded minimally to the treatment and required support in daily activities (mRS score of 4) were defined as having a severe deficit.

This study was approved by the institutional review boards at Keio University and the National Center of Neurology and Psychiatry. Statistical analyses were performed using StatView version 5.0 statistical software (SAS Institute, Inc).

RESULTS

Figure 1 shows the distribution of periods between disease onset and the first examination. We divided 27 patients with anti-SRP myopathy into 2 subtypes (subacute and chronic forms) based on the clinical course. Of the 27 patients with anti-SRP myopathy in our study, 5 (19%) were considered to have the chronic form. The patients' demographic and clinical features are compared between those with the subacute and chronic forms (**Table 1**). Disease onset occurred at a younger age in those with the chronic form than in those with the subacute form (mean age, 15.4 vs 52.4 years, respectively; $P < .001$). No patients with the chronic form had a clear clinical history of antecedent infection, whereas 3 patients (14%) with the subacute form had antecedent infection. Despite a previous report,[5] seasonal occurrence was not clear in our series. Disease progression of the subacute form was usually rapid, and the mean duration between disease onset and the first examination was 3.1 months. In particular, 3 patients showed rapid disease progression in 2 to 3 weeks. In contrast, patients with the chronic form showed significantly slower progression, and the mean duration between disease onset and the first examination was 10.2 months ($P = .001$).

In our series, asymmetrical muscle involvement was seen in 2 patients, whereas the other 25 patients showed proximal-dominant symmetrical limb muscle weakness. Lower limbs were more severely affected than upper limbs. All 5 patients with the chronic form and about half of the patients with the subacute form showed severe muscle weakness and atrophy at the first examination. Several reports emphasized that dysphagia, but not dysarthria, was observed at a high frequency in 43% to 75% of patients with anti-SRP myopathy.[3,5,7] In our series, 7 patients (26%) had dysphagia and 3 (11%) reported it as the initial symptom. Previous reports also showed a high frequency of cardiac involvement,[2,5] while only 1 patient in our series had arrhythmias, which did not require treatment. Respiratory muscle involvement was detected in 3 patients. Myalgia was noted in 9 patients (36%) and tended to precede muscle weakness. Extramuscular manifestations were observed only in patients with the subacute form. Skin rash and interstitial lung disease, which were clinically suggestive of dermatomyositis, were observed in 2 and 4 patients, respectively. Serum CK levels were markedly elevated to more than 1000 IU/L (to convert to microkatals per liter, multiply by 0.0167) in all 27 patients; however, there was no difference between the subacute and chronic forms. Other autoantibodies were found in 6 patients with the subacute form, including Ro/SSA (3 patients), Th/To (1 patient), ribosome (1 patient), and U1RNP (1 patient).

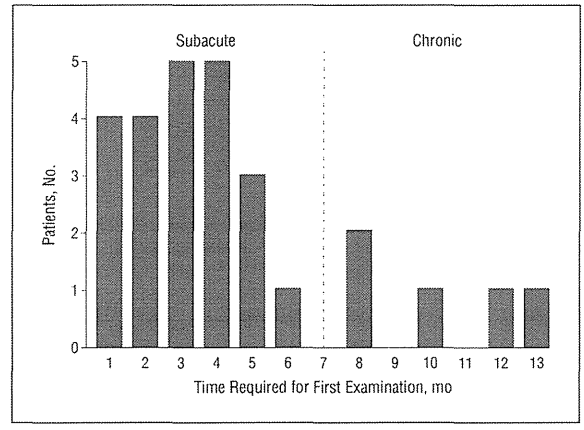

Figure 1. Period between disease onset and the first examination in 27 patients with anti–signal recognition particle myopathy. They were divided into 22 patients with the subacute form and 5 patients with the chronic form based on the clinical course.

All 27 patients were treated with oral prednisolone (1 mg/kg/d). Half of the patients were treated with additional immunosuppressive agents, including methotrexate (n = 5), azathioprine (n = 4), tacrolimus (n = 2), cyclophosphamide (n = 1), and cyclosporine (n = 1), or with intravenous immunoglobulin (n = 6). Although some patients required 2 to 3 months to respond to treatment, the patients with anti-SRP myopathy did not always respond poorly. The combination of oral prednisolone and intravenous immunoglobulin appears to be most effective for patients with the subacute form as the initial treatment. The neurological outcomes showed that 10 patients (45%) with the subacute form recovered. In contrast, all 5 patients with the chronic form had more severe neurological outcomes compared with the 22 patients with the subacute form ($P = .008$) (**Figure 2**).

Table 1. Comparison of Clinical Features Between Subacute and Chronic Forms of Anti–Signal Recognition Particle Myopathy

Clinical Feature	Patients, No. (%)		*P* Value
	Subacute (n = 22)	Chronic (n = 5)	
Age at onset, mean (range), y	52.4 (14-82)	15.4 (5-32)	<.001[a]
Female	12 (55)	3 (60)	.78[b]
Antecedent infection	3 (14)	0	.93[b]
Time required for first examination, mean (range), mo	3.1 (1-6)	10.2 (8-13)	.001[a]
Muscle weakness			
Arms < legs	16 (73)	3 (60)	.98[b]
Arms > legs	6 (27)	2 (40)	.98[b]
Severe involvement	11 (50)	5 (100)	.12[b]
Laterality	1 (5)	1 (20)	.80[b]
Facial muscle involvement	1 (5)	1 (20)	.80[b]
Bulbar sign	6 (27)	1 (20)	.81[b]
Cardiac involvement	1 (5)	0	.80[b]
Respiratory failure	3 (14)	1 (20)	.73[b]
Neck weakness	9 (41)	4 (80)	.27[b]
Muscle atrophy	10 (45)	5 (100)	.08[b]
Myalgia	8 (36)	1 (20)	.86[b]
Extramuscular involvement			
Fever	4 (18)	0	.73[b]
Skin rash	2 (9)	0	.80[b]
Arthritis	1 (5)	0	.80[b]
Raynaud phenomenon	1 (5)	0	.80[b]
Interstitial lung disease	4 (18)	0	.73[b]
Associated disorder			
Cancer	1 (9)	0	.80[b]
Rheumatic disorder	1 (9)	0	.80[b]
Serum creatine kinase, mean (range), IU/L	6101 (1149-15 585)	4190 (2465-5725)	.08[a]

SI conversion factor: To convert serum creatine kinase to microkatals per liter, multiply by 0.0167.
[a] Statistical analysis by *t* test.
[b] Statistical analysis by χ^2 test.

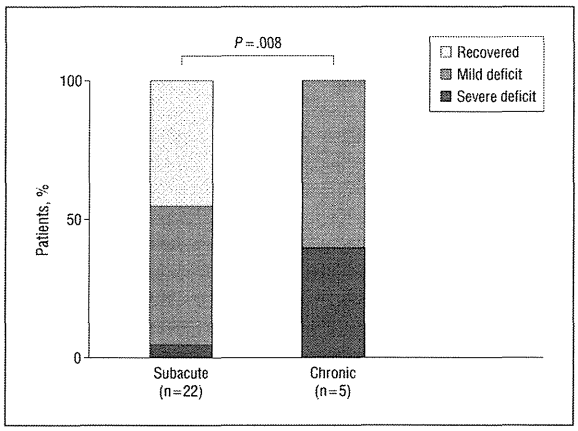

Figure 2. Neurological outcomes were assessed using the modified Rankin Scale[12] with some modifications and were compared between subacute and chronic forms of anti–signal recognition particle myopathy. The neurological outcomes were divided into recovered, mild deficit, and severe deficit. Differences between the groups were analyzed with the Mann-Whitney test. Five patients with the chronic form showed more severe outcomes than 22 patients with the subacute form ($P = .008$).

Detailed clinical features of 5 patients with the chronic form are summarized in **Table 2**. All patients had severe muscle weakness and marked atrophy in all 4 limbs and the trunk. Two patients (patients 2 and 5) noticed arm muscle weakness as the initial symptom. Importantly, scapular winging was noted in 2 patients (patients 2 and 3) at the first examination and was suspected to involve facioscapulohumeral muscular dystrophy. The serum CK level was decreased after treatment in patients with the chronic form, but muscle weakness gradually progressed and recovery of muscle strength was delayed. Three patients (patients 1, 2, and 3) became unable to walk independently, and 1 (patient 3) required mechanical ventilation. Because muscle biopsies were not suggestive of inflammatory myopathy, 1 patient (patient 3) was treated for only 3 months and 2 (patients 1 and 2) were treated after the detection of anti-SRP antibody. Of these younger patients, 2 (patients 2 and 3) became severely disabled, whereas the other 2 (patients 4 and 5) were treated soon after the muscle biopsy and responded partially to treatment.

COMMENT

There are 2 methods for detecting anti-SRP antibodies: the RNA immunoprecipitation assay we used and an immunoassay using the signal peptide–binding 54-kDa subunit of SRP (SRP54) as the antigen. Because SRP54 is regarded as the main antibody target, the immunoassay using SRP54 is easily conducted and the antibody level is also available.[1,2,14] However, epitopes of anti-SRP antibodies may also be located in other subunits of SRP proteins or 7SL-RNA.[7,15] In contrast, RNA immunoprecipitation assay, the standard method for detection of

Table 2. Clinical Features of 5 Patients With the Chronic Type of Anti–Signal Recognition Particle Myopathy

Feature	Patient No. 1[a]	2[a]	3[a]	4	5
Sex	F	F	M	F	M
Age at onset	5 y 9 mo	9 y 8 mo	10 y 2 mo	20 y 10 mo	32 y 9 mo
Initial symptoms	Frequent falls	Difficulty raising arm	Difficulty running fast	Difficulty climbing stairs	Difficulty raising his child
Weakness and atrophy	Proximal limbs (U < L); trunk	Proximal limbs (U > L); trunk; scapular winging; left dominant; myalgia	Proximal limbs (U < L); trunk; scapular winging; facial, bulbar; respiratory	Proximal limbs (U < L); trunk	Proximal limbs (U > L); trunk; strenocleidomastoideus
Serum creatine kinase, IU/L	4629	2467	4180	3951	5725
Muscle images	Atrophy in proximal limbs and trunk	Left-dominant atrophy and edematous change in proximal limbs and trunk	Atrophy and edematous change in proximal limbs and trunk	Atrophy and edematous change in proximal limbs and trunk	Atrophy and edematous change in proximal limbs and trunk
Age at muscle biopsy	6 y 5 mo	10 y 4 mo	11 y 3 mo, 16 y 6 mo	21 y 8 mo	33 y 9 mo
Muscle biopsy					
Variation in fiber size	Scattered	Scattered	Marked	Marked	Marked
Fiber necrosis and regeneration	Moderate	Marked	Marked	Scattered	Marked
Lymphocyte infiltration	None	None	None	None	Perivascular
Endomysial fibrosis	Minimal	Mild	Marked	Minimal	Mild
Age at anti-SRP antibody detection	7 y 4 mo	10 y 9 mo	32 y 6 mo	21 y 10 mo	34 y 3 mo
Age at treatment start	7 y 4 mo	10 y 9 mo	11 y 6 mo	21 y 8 mo	33 y 9 mo
Treatment	PSL, MTX, MPR	PSL, MTX, IVCY, AZA, tacrolimus	PSL (3 mo)	PSL, MPR	PSL, MTX, IVIg, tacrolimus
Age at final follow-up	9 y 3 mo	13 y 10 mo	34 y 8 mo	23 y 3 mo	35 y 6 mo
Response and neurological outcome	Partial response; progression for 2 y; relapse; MMT grade 4; Gowers sign	Minimal response; progression for 2 y; MMT grade 2-3; walking 20 m; difficulty in holding dishes	No response; progression for 3 y; recovered from mechanical ventilation; MMT grade 2-3; wheelchair use	Partial response; progression for 1 y; MMT grade 4	Partial response; progression for 1.5 y; weakness recovered; relapse

Abbreviations: AZA, azathioprine; IVCY, intravenous cyclophosphamide; IVIg, intravenous immunoglobulin; L, lower; MMT, manual muscle strength; MPR, high-dose methylprednisolone sodium succinate; MTX, methotrexate; PSL, prednisolone; SRP, signal recognition particle; U, upper.

SI conversion factor: To convert serum creatine kinase to microkatals per liter, multiply by 0.0167.

[a]These patients were previously described.[9,10]

anti-SRP antibodies, has advantages in sensitivity and specificity.[1,2,4,6,9,11] The RNA immunoprecipitation assay can recognize the conformational epitopes of SRP, although the titer of antibodies is not available. Many studies showed that anti-SRP antibodies were principally specific to myositis or necrotizing myopathy except in a few patients with systemic sclerosis or rheumatoid arthritis.[1,2,4,6,9,11] In regard to myopathies, we demonstrated that anti-SRP antibody was not detected in patients with various types of muscular dystrophy, and it was useful for the differential diagnosis of myopathies using RNA immunoprecipitation assay.[9]

Anti-SRP myopathy can show a wider variety of clinical symptoms than was previously considered. When weakness progresses rapidly, within 2 to 3 weeks, with extremely high serum CK levels (>10 000 IU/L), acute rhabdomyolysis should be differentiated.[8] When patients experience progressive weakness within 2 to 6 months[2-7] accompanied by interstitial lung disease, skin rash, or associated rheumatic disorders, polymyositis or dermatomyositis should be considered. Because skin rash is observed in approximately 10% of cases of anti-SRP myopathy in the present and previous studies,[5] anti-SRP antibodies may be also detected in patients clinically diagnosed as having dermatomyositis. In fact, Hamaguchi et al[16] reported that anti-SRP antibodies were detected in 7 of 376 patients (2%) with dermatomyositis using a similar detection method.

In our series, 5 of 27 patients with anti-SRP myopathy (19%) showed chronic progressive muscle involvement. The mean age at onset in these 5 patients was significantly younger than that of the patients with the subacute form, and patients with the chronic form showed severe weakness and atrophy in limbs and trunk muscles as well as poorer outcomes. It was speculated that the poor outcome may be partially ascribed to the delay of the first examination or anti-SRP antibodies detection. Importantly, these clinical features may indicate the possibility of muscular dystrophy rather than inflammatory myopathy,[8-10] although the disease progression was faster than occurs in muscular dystrophy. In fact, facioscapulohumeral muscular dystrophy was initially suspected in 2 patients owing to prominent shoulder-girdle weakness.[9,10]

It is well known that anti-SRP myopathy is usually resistant to treatment, resulting in severe disablement.[2-4,6,7] However, our observation suggested that patients with the subacute form had relatively good neurological outcomes. Early diagnosis by screening for anti-SRP antibodies is important for choosing intensive immunotherapy, which might contribute to better outcomes. In this regard, Hengstman et al[5] reported that the response to treatment for patients with anti-SRP myopathy did not differ significantly from that of myositis without anti-SRP antibodies. They reported that 75% of patients with anti-SRP myopathy could walk without any assistance after treatment. The severe outcomes of anti-SRP myopathy described in the previous studies may be attributable partly to results for patients with the chronic form. Rituximab therapy is potentially effective for patients with the chronic form.[7] Based on these findings, it may be useful to divide patients by disease progression to predict the neurological outcome.

An apparent question about the relationship between anti-SRP antibodies and muscle involvement is whether the anti-SRP antibodies themselves have any pathogenic effect against muscle. This hypothesis may be supported by several lines of data: (1) anti-SRP antibodies purified from patients' serum samples can inhibit the in vitro translocation of secretory proteins into endoplasmic reticulum[17]; (2) the levels of anti-SRP54 autoantibodies are closely associated with the levels of myolysis[14]; and (3) the removal of anti-SRP antibodies by plasma exchange improves muscle strength.[14,18] Nevertheless, the causal relationship between anti-SRP antibodies and muscle involvement is still not established, and further experiments such as passive transfer to animals are necessary to elucidate the pathogenesis of anti-SRP antibodies.

In conclusion, anti-SRP myopathy can show quite variable disease progression and neurological outcomes.

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Original article

The diagnostic utility of anti-melanoma differentiation-associated gene 5 antibody testing for predicting the prognosis of Japanese patients with DM

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Abstract

Objective. Interstitial lung disease (ILD), especially rapidly progressive ILD (RPILD), is a major poor prognostic factor in patients with DM. We investigated the association of anti-melanoma differentiation-associated gene 5 (MDA5) antibody (Ab) with clinical characteristics and mortality in Japanese patients with DM.

Methods. Seventy-nine DM patients, comprising 58 classic DM and 21 clinically amyopathic DM (CADM) patients, were enrolled. Serum Abs were screened by immunoprecipitation assays, and an immunosorbent assay (ELISA) was used for MDA5. The relationships of clinical characteristics and mortality with each Ab were investigated.

Results. Anti-MDA5 Ab was detected in 17 patients. Anti-clinically amyopathic DM 140 kDa polypeptide Abs (anti-CADM-140 Abs) were found in 16 of the 17 anti-MDA5 Ab^+ patients. Skin ulcers, palmar papules, CADM, RPILD and mediastinal emphysema were widely distributed in anti-MDA5 Ab^+ patients. Mortality at 6 months as well as 5 years was also significantly higher in anti-MDA5 Ab^+ patients than in anti-MDA5 Ab^- patients. In a multivariable Cox regression analysis, mortality was independently associated with anti-MDA5 Ab (relative hazard 6.33; 95% CI 1.43, 28.0). All of the deaths in anti-MDA5 Ab^+ patients were attributed to respiratory failure of RPILD; however, RPILD did not worsen in any of the anti-MDA5 Ab^+ patients who survived the first 6 months.

Conclusion. The presence of anti-MDA5 Ab identifies the characteristic skin, musculoskeletal, pulmonary and prognostic features in patients with DM. In addition, anti-MDA5 Ab seems to predict a group of patients with CADM-complicated fatal RPILD.

Key words: anti-MDA5 Ab, CADM, RPILD.

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CLINICAL SCIENCE

Introduction

A number of autoantibodies can be detected in the sera of patients with DM, some of which are specific to DM and are known as myositis-specific autoantibodies (MSAs). Moreover, these autoantibodies are closely associated with clinical manifestations of DM, such as symptoms, complications, reactivity to therapy and prognosis [1].

In recent years, the autoantibodies found in patients with inflammatory myopathies have been mainly classified into several types by immunoprecipitation assays: anti-aminoacyl-tRNA synthetase antibodies (anti-ARS Abs), Abs to the signal recognition particle (anti-SRP Abs), anti-Mi2 Abs, PM/Scl-100 Abs and PM/Scl-75 polypeptides Abs (anti-PM-Scl Abs), anti-clinically amyopathic DM 140 kDa polypeptide Abs (anti-CADM-140 Abs), anti-155/140 kDa polypeptide Abs (anti-p155/140 Abs) and autoantibodies to a 142 kDa protein (anti-MJ Abs). These autoantibodies are strongly associated with the clinical presentation [2–6]. In this regard, we have reported a high frequency of rapidly progressive interstitial lung disease (RPILD) and clinically amyopathic DM (CADM) associated with anti-CADM-140 Abs [7, 8]. Recently RNA helicase encoded by melanoma differentiation-associated gene 5 (MDA5) was identified as a major autoantigen in patients with CADM, which is targeted by anti-CADM-140 Abs [9, 10].

Gono *et al.* [11] have also recently reported that anti-MDA5 Ab predicts a fatal outcome in patients with DM combined with RPILD; however, the long-term prognosis and other clinical characteristics of anti-MDA5 Ab$^+$ DM patients remain to be elucidated. In the present study we have tried to investigate the clinical value of anti-MDA5 Ab for DM patients in a single cohort.

Patients, materials and methods

Patients

Sera samples were obtained from 79 patients with DM who were undergoing medical treatment at the Graduate School of Biomedical Sciences, Nagasaki University, from September 1999 to August 2010, and were stored at −20°C until use. Most of the sera samples were obtained at the first visit so the interval from initiation of therapy was minimal. We collected the data from all of the DM patients examined in our department. Twenty-one patients did not fulfill Bohan and Peter's criteria [12, 13] but fulfilled Sontheimer's criteria (CADM) [14, 15] because of the absence of clinical skeletal muscle symptoms and the presence of persistent clinical DM skin features. Clinical manifestations, laboratory data, radiographic data and the presence of internal malignancies were extracted from medical records and verified by T.K., N.I. and K.F. The patients were diagnosed with ILD according to the results of chest X-ray and high-resolution chest CT, reported by Japanese board-certified radiologists. All of the subjects underwent routine examination of internal malignancies and chest radiography. A subset of patients with RPILD was defined as those presenting with progressive dyspnoea and progressive hypoxaemia, and a worsening of interstitial change on chest radiography within 1 month from the onset of respiratory symptoms, as described previously [2]. A signed consent form to participate in the study, which was approved by the Institutional Review Board of Nagasaki University, was obtained from each patient.

Immunoprecipitation and ELISA

MSAs, including anti-CADM-140 Abs, anti-ARS Abs and anti-155/140 Abs, were detected by immunoprecipitation assays using extracts of leukaemia cell line K562, as described previously [3]. Interpretation of the results of immunoprecipitation was undertaken without knowledge of patients' clinical status. An ELISA system using recombinant MDA5 as an antigen source was performed as described previously [10]. All samples were examined in duplicate, and the Ab units were calculated from the optical density at 450 nm, using a standard curve obtained from serial concentrations of a serum sample containing a high titre of anti-CADM-140 Abs. The cut-off level was set at 8.0 U, based on 10 S.D. above the mean value obtained from 32 healthy control sera. Interpretation of the results of ELISA was undertaken without knowledge of the clinical status of the patients and the results of immunoprecipitation assays.

Statistical analysis

Fisher's exact probability test and the Mann–Whitney U-test were used to compare the differences. We also examined the cumulative survival rates from the first visit to the hospital with DM-related symptoms up to 5 years by the multivariate Cox proportional hazard model adjusted for patient age at symptoms onset, gender, with or without CSs and with or without immunosuppressants. A $P < 0.05$ was considered significant.

Results

Clinical characteristics of anti-MDA5 Ab$^+$ patients

Table 1 summarizes the 17 DM patients with anti-MDA5 Ab and the 62 DM patients without anti-MDA5 Ab. There were 21 patients with CADM in the present study and we have found that anti-MDA5 Ab is detected in 14 of 21 patients. In this group, 11 of 14 (79%) patients had complicated RPILD and 7 (50%) patients died. Our present data confirm the recent publications regarding the characteristics of anti-MDA5 Ab$^+$ patients, including the CADM, RPILD, low CK, high ferritin and high mortality found in these patients [11]. Since anti-MDA5 Ab is mostly attributed to anti-CADM-140 Abs, a high prevalence of palmar papules and mediastinal emphysema, which has been reported as typical of anti-CADM-140 Abs$^+$ DM patients by our group [7], was also preferentially found in anti-MDA5 Ab$^+$ patients. The present finding that skin ulcers are highly prevalent in anti-MDA5 Ab$^+$ patients is new, however. Muscle biopsy or lung biopsy was not performed. Skin biopsies were taken from eight patients positive for anti-MDA5 Abs, and six patients were

TABLE 1 Comparison of clinical manifestations between patients with anti-MDA5 Ab and patients without anti-MDA5 Ab

Variable	Anti-MDA5 Ab		
	Positive ($n = 17$)	Negative ($n = 62$)	*P*-value
Age at onset, years	55.5 (13.0)	55.3 (15.0)	0.27
Female, *n* (%)	15 (88)	37 (60)	0.056
Skeletal muscle and skin features			
Muscle weakness, *n* (%)	4 (24)	38 (62)	**0.005**
Gottron's sign, *n* (%)	13 (76)	32 (52)	0.07
Ulcer region, *n* (%)	10 (59)	7 (12)	**0.00007**
Heliotrope rash, *n* (%)	8 (47)	23 (39)	0.56
Palmar papules, *n* (%)	11 (65)	13 (22)	**0.0014**
Periungual erythema, *n* (%)	10 (59)	24 (41)	0.2
Clinical diagnosis			
CADM, *n* (%)	14 (82)	7 (11)	**4.2×10^{-9}**
Pulmonary involvement and malignancy			
ILD, *n* (%)	16 (94)	37 (61)	**0.008**
RPILD, *n* (%)	12 (71)	4 (7)	**9.8×10^{-9}**
Mediastinal emphysema, *n* (%)	6 (35)	1 (2)	**2.1×10^{-5}**
Malignancies, *n* (%)	0 (0)	6 (10)	0.17
Laboratory data			
CPK, IU/l	173 (53–468)	905 (107–1607)	**0.00024**
KL-6, U/ml	1361 (825–1903)	1040 (345–1510)	0.36
Ferritin, ng/ml	1365 (894–1751)	180 (90–244)	**0.016**
Therapy			
Maximum PSL, mg/day	40 (35–50)	40 (22.5–50)	0.99
Immunosuppressant, *n* (%)	16 (94)	29 (47)	0.17
Outcome			
Death, *n* (%)	7 (41)	3 (5)	**6.6×10^{-6}**
MSA profile			
Anti-140 Ab positive, *n* (%)	16 (94)	0 (0)	**3.76×10^{-15}**
Anti-155/140 Ab positive, *n* (%)	0 (0)	7 (11)	0.35
Anti-ARS Ab positive, *n* (%)	0 (0)	30 (48)	**0.002**
Autoantibody negative	1 (6)	25 (40)	**0.005**
Anti-MDA5 Ab titre	230 (22–448)	1.3 (1.1–1.9)	**1.62×10^{-10}**

Ages are presented as mean (S.D.) values, while laboratory markers are medians (interquartile range). *P*-values were established using Fisher's exact test or the Mann–Whitney U-test. Bold indicates significant values. CPK: creatinine phosphokinase; PSL: prednisolone.

diagnosed pathologically with dermatitis consistent with DM. One patient revealed only mild mucin deposition, and another revealed only hyperpigmentation. A potential limitation of the present study is the fact that biopsies were taken from only a small number of patients. EMG was performed in one anti-MDA5 Ab$^+$ patient, revealing myogenic conversion consistent with myositis. Only one patient was found to have preceding ILDs among anti-MDA5 Ab$^+$ patients. Skin manifestations preceded ILDs in the other patients. We showed the typical images about mediastinal emphysema, palmar pustule and regional ulcers in anti-MDA5 Ab$^+$ patients with CADM (Fig. 1). In the frequency of cancer, anti-MDA5 Ab$^+$ patients have no malignancy (0/17), whereas 6 of 62 (10%) patients in anti-MDA5 Ab$^-$ group were complicated malignancies. Anti-155/140 Abs were found in all six patients with cancer. We confirmed the profile of autoantibodies regarding the presence or absence of anti-MDA 5 Ab: namely, all DM patients positive for anti-ARS Abs, anti-155/140 Abs and other types of autoantibodies were among the anti-MDA5 Ab$^-$ group. There was no overlap between anti-MDA5 Ab and any other types of autoantibodies. Immunoprecipitation of anti-CADM-140 Abs from patients with anti-MDA5 Ab is shown in Fig. 2.

Survival rate of anti-MDA5$^+$ patients

Ten (12%) patients died within 5 years from the first treatment. The cumulative 6-month survival rates were 57.4 and 98.4% for DM with anti-MDA5 Ab and those without anti-MDA5 Ab, respectively (Fig. 3). The survival rates from the first visit to our hospital after adjusting for age, gender, with or without CSs and with or without immunosuppresants were significantly different between each subset ($P = 0.0151$). The first visit to our hospital was almost identical to the diagnosis of each patient. The presence of anti-MDA5 Ab was independently associated with mortality (relative hazard 6.33; 95% CI 1.43, 28.0) in a multivariable Cox regression model that included patient age at onset, gender, with or without CSs and with or without immunosuppresants. We have tried to compare

FIG. 1 Typical clinical manifestations of patients with anti-MDA5 Ab. The palmar pustules (**A**) were mainly located near the MCP and PIP joints (arrows) and multiple ulcer regions (**B**) were also observed. Chest CT scan (**C**) shows mediastinal emphysema in the middle of the chest cavity (arrows).

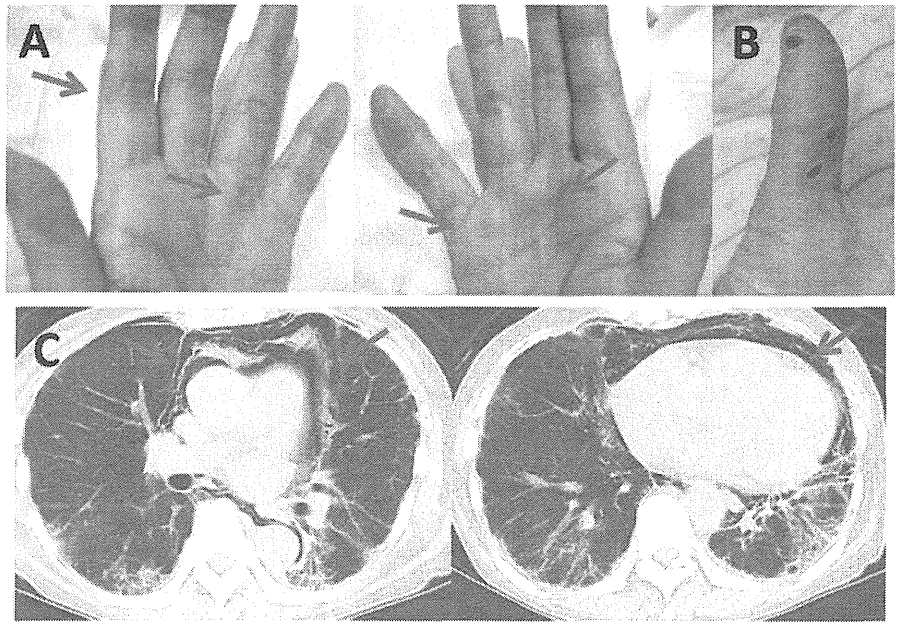
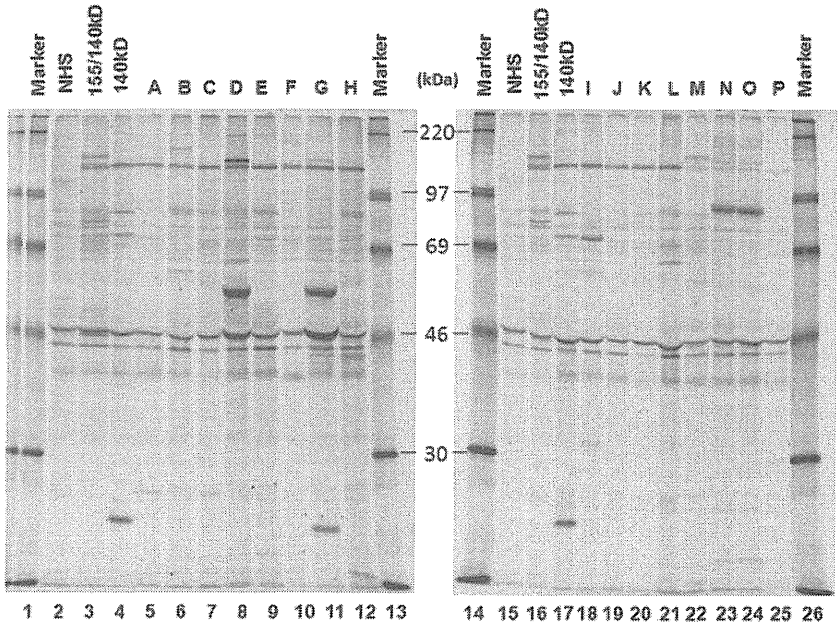
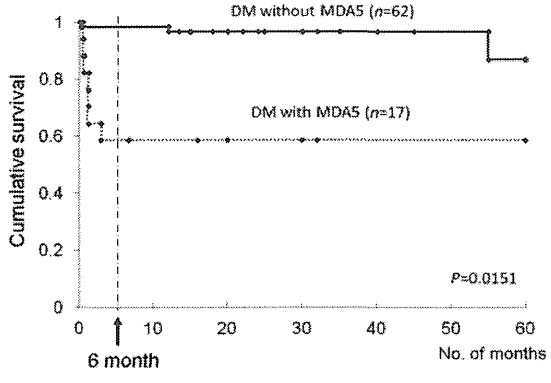


FIG. 2 Immunoprecipitation with anti-CADM-140 Ab from the 35S-labelled K562 cell extract. Lanes 5–12 and 18–25 show the results with anti-CADM-140-positive sera from DM patients with anti-MDA5 Ab$^+$ (A–P). The results of the prototype sera of anti-155/140 Abs and anti-CADM-140 Abs are also shown (lanes 3, 16 and 4, 17, respectively). One sera of an anti-MDA5 Ab$^+$ patient immunoprecipitated not anti-CADM-140 Abs, but anti-U1-RNP Ab, which was deleted from Fig. 2.



the variables within anti-MDA5 Ab$^+$ DM patients who were alive or dead and found that the regime of therapy was not different between two groups although the PaO_2/FiO_2 and serum CPK levels were higher in the former. The value of anti-MDA5 Ab is significantly lower in the former (Table 2). All the deaths in the anti-MDA5 Ab$^+$ patients were attributed to respiratory failure of RPILD. However, importantly, there was no acute exacerbation or progressive worsening of ILD by CT images after initial treatments in any of the anti-MDA5 Ab$^+$ patients. In fact, all of the deaths of anti-MDA5 Ab$^+$ patients occurred within the first 6 months (Fig. 3). In addition, no patient required home oxygen therapy after discharge among anti-MDA5 Ab$^+$ patients who were alive during the first 6 months. We showed a short case presentation describing a patient with CADM positive for anti-MDA5 Ab. A 60-year-old female developed erythemas on the upper eyelids, fingers and elbows in July 2005. Three months later she developed exertional dyspnoea. A CT scan revealed interstitial lung shadow (Fig. 4A). We measured anti-CADM-140 Ab levels and anti-MDA5 Ab levels, which were both positive (anti-140 kDa Abs were detected by immunoprecipitation assay, and the titre of anti-MDA5 Abs was 544.109 U). She has been treated at our outpatient department and is in a stable condition (Fig. 4B).

FIG. 3 The adjusted cumulative survival rates in the presence or absence of anti-MDA5 Ab. The cumulative survival rates from the first visit to the hospital with DM-related symptoms up to 5 years were examined as described in the text. Survival rate of anti-MDA5 Ab$^+$ patients was significantly low compared with that of anti-MDA5 Ab$^-$ patients. $P = 0.0151$, between the two groups.



Discussion

Other Japanese groups recently identified the characteristics of anti-MDA5 Ab$^+$ DM patients [11]. Our present data confirmed their findings. Additionally, we have shown some new characteristics of these patients, such as high frequencies of palmar papules, skin ulcers and mediastinal emphysema, as well as no overlapping of other types of autoantibodies. These data may help physicians to recognize features of anti-MDA5 Ab$^+$ patients among DM patients. Since physicians are urged to start intense immunosuppressive therapy early for anti-MDA5 Ab$^+$ DM patients, this information may be clinically indispensable.

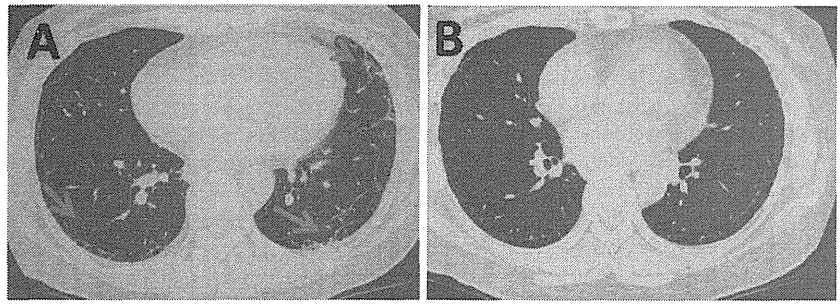
Although the prognosis of anti-MDA5 Ab$^+$ patients was worse than that of anti-MDA5 Ab$^-$ patients, none of the surviving anti-MDA5 Ab$^+$ patients experienced acute exacerbation or progressive worsening of ILD after the initial treatment. This is quite different from anti-MDA5 Ab$^-$ patients, since ILD recurred in several of these patients and death ensued during long-term follow-up (Fig. 3). One of the characteristics of anti-MDA5 Ab$^+$ patients is hyperferritinaemia [11, 16]. There are many

TABLE 2 Comparison of clinical parameters between alive and dead anti-MDA5 Ab$^+$ patients

Variable	Anti-MDA5 Ab positive ($n = 17$)		
	Alive ($n = 10$)	Dead ($n = 7$)	*P*-value
Age at onset, years	52 (42–58.5)	59 (53–70)	0.051
Female, *n* (%)	9 (90)	6 (86)	1.00
Ulcer region, *n* (%)	5 (50)	5 (71)	0.70
Palmar papules, *n* (%)	7 (70)	5 (71)	1.00
CPK, IU/l	208 (90.3–864)	169 (33.5–359)	**0.014**
Anti-MDA5 Ab titre	168 (16.3–436)	230 (76.0–478)	**0.032**
PaO_2/FiO_2 before treatment, mmHg	395 (370–462)	203 (114–240)	**0.027**
Therapy			
Steroid pulse therapy, *n* (%)	5 (50)	7 (100)	0.09
CYC, *n* (%)	4 (40)	4 (57)	0.84
Oral calcinurin inhibitor, *n* (%)	6 (60)	7 (100)	0.18
I.V. calcinurin inhibitor, *n* (%)	1 (10)	3 (43)	0.32

Ages are presented as mean (S.D.) values, while laboratory markers are medians (interquartile range). *P*-values were established using Fisher's exact test or the Mann–Whitney U-test. Bold indicates significant values. PaO_2: partial pressure of arterial oxygen; FiO_2: fractional inspired oxygen concentration.

FIG. 4 A chest CT scan before and after treatment. A reticular shadow was revealed in the lower lung field (**A**) and it improved 4 years after disease onset (**B**). Arrows indicate the region that improved with treatment.



reports evaluating hyperferritinaemia in patients with autoimmune diseases [17]. The highest ferritin levels in autoimmune disorders are typically seen in patients with macrophage activation syndrome (MAS), often associated with adult-onset Still's disease (AOSD) [18]. It is well known that many viruses produce double-stranded (ds) RNA that can be recognized by two major arms of the innate immune system: the toll-like receptors (TLRs) and the Rig-I-like receptors (RLRs). MDA5 is a member of the RLR family that recognizes dsRNA within the cytosolic compartment and induces the production of inflammatory cytokines and cell surface molecules involved in the anti-viral response [19]. Considering that MAS could be induced by various infectious agents [20], and given the critical role of MDA5 in innate immune defence against viruses, one hypothesis is that the production of anti-MDA5 Ab is an epiphenomenon during virus infection that is associated with the onset of CADM and RPILD; namely, infection of the skin and lung epithelium by certain viruses. In general, innate immune responses do not recur; therefore we have not found exacerbation of ILD during the follow-up periods of anti-MDA5 Ab^+ DM patients.

Most patients with ILD-complicated DM appear to be well controlled by CSs and immunosuppressants [21]. In contrast, patients with RPILD observed in DM were resistant to a variety of treatments [22, 23]. We have introduced CSs, cyclophosphamide and calcineurin inhibitor to anti-MDA5 Ab^+ patients with RPILD. We could not find any significant difference in therapy between alive and dead patients. PaO_2/FiO_2, serum CPK level and the value of anti-MDA5 Ab before treatment were prognostic factors. We showed the significance of the duration of preceding symptoms in patients positive for anti-MDA5 Abs. Although we do not have any definitive evidence, shorter duration of preceding symptoms to treatment could lead to better outcomes (supplementary Table 1, available as supplementary data at *Rheumatology* Online). Thus it is recommended that anti-MDA5 Ab^+ patients who have typical CADM with signs of ILD be treated promptly with the combination of CSs, cyclophosphamide and calcineurin inhibitor.

In conclusion, the measurement of anti-MDA5 Ab by ELISA enables us to predict the prognosis of patients with CADM-complicated fatal RPILD. The characteristics of anti-MDA5 Ab^+ DM patients could be explained by the nature of MDA5 in innate immune responses to viruses. A multicentre, prospective study is warranted to confirm our results.

Rheumatology key messages

- Anti-MDA5 Ab is associated with characteristic pulmonary and skin involvement in patients with DM.
- Anti-MDA5 Ab predicts patients with CADM complicated by RPILD.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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Original article

Heparin-dependent and -independent anti-platelet factor 4 autoantibodies in patients with systemic lupus erythematosus

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Abstract

Objective. Antibodies that recognize complexes formed by platelet factor 4 (PF4) and heparin are involved in the pathogenesis of heparin-induced thrombocytopenia (HIT). This study was undertaken to investigate the prevalence and clinical correlations of anti-PF4 autoantibodies in patients with SLE.

Methods. We studied 118 patients with SLE, 78 with primary immune thrombocytopenia (ITP), 27 with primary APS, 2 with HIT (as positive controls) and 47 healthy controls. Heparin-dependent and -independent anti-PF4 antibodies were measured with an ELISA. Antibody binding was confirmed to be heparin-dependent when inhibited by the presence of a high concentration of heparin. Pathogenic anti-PF4 antibody was assessed by serotonin-release assay.

Results. Heparin-dependent anti-PF4 antibodies were detected in 11 SLE (9%) and 2 primary ITP (3%) patients, but at much lower levels than in HIT patients. In serotonin-release assays, only the HIT sera induced platelet activation *in vitro*. Heparin-independent anti-PF4 antibodies were detected in 17 SLE patients (14%). There was no correlation between the levels of heparin-dependent and -independent anti-PF4 antibodies. Cross-reactivity between these two antibodies was not detectable by ELISA competitive assay. Heparin-dependent anti-PF4 antibodies were associated with thrombocytopenia and IgM aCLs ($P = 0.007$ for both comparisons), while heparin-independent anti-PF4 antibody levels were correlated with SLE disease activity index ($P = 0.0005$). None of the SLE patients with anti-PF4 antibodies had previous heparin exposure.

Conclusion. PF4 is an autoimmune target in SLE patients. Heparin-dependent and -independent anti-PF4 autoantibodies may be involved in different aspects of pathophysiology of SLE.

Key words: autoantigens and autoantibodies, systemic lupus erythematosus and autoimmunity, haematopoietic, laboratory diagnosis, immunological techniques.

Introduction

Heparin-induced thrombocytopenia (HIT) is an immune-mediated disorder that can develop during anti-coagulation therapy with heparin, particularly unfractionated heparin [1]. Approximately 25% of patients with HIT paradoxically develop arterial and/or venous thrombosis. HIT is associated with antibodies that recognize oligomeric complexes formed between platelet factor 4 (PF4) and heparin or endogenous heparinoids, such as GAGs [2]. There is increasing evidence that immune complexes formed by IgG antibodies and the PF4–heparin complex bind to FcγRIIA receptors on platelets, activating the platelets and accelerating the coagulation pathway by generating thrombin [2]. These heparin-dependent anti-PF4 antibodies recognize neoepitopes formed on the PF4–heparin complex, and are a hallmark of HIT. Recently heparin-dependent anti-PF4 antibodies were also documented in individuals without clinical HIT or previous heparin exposure [3–7]. Several studies have

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