demonstrated that heparin-dependent anti-PF4 antibodies are present in 4–15% of patients with SLE or APS [5, 6]; however, Pauzner *et al.* [8] reported that the majority of such patients actually had antibodies reactive with PF4 alone, or heparin-independent anti-PF4 antibodies. These findings suggest that PF4 may be targeted by autoantibody responses in systemic autoimmune diseases. Despite the definitive role of heparin-dependent anti-PF4 antibodies in the pathogenesis of HIT, the clinical relevance of naturally occurring anti-PF4 autoantibodies, heparin-dependent or -independent, in SLE patients remains unclear. Some authors have postulated that heparin-dependent anti-PF4 antibodies may represent an additional prothrombotic risk for APS [7, 9], whereas another report failed to reproduce this association [6].

In this study we examined heparin-dependent and -independent anti-PF4 antibodies in patients with SLE, primary APS and primary immune thrombocytopenia (ITP), conditions that share clinical features in whole or in part with HIT, and evaluated associations between antibody specificities and clinical characteristics, including thrombocytopenia and thromboembolism.

Materials and methods

Patients and controls

We studied 118 patients with SLE, 78 with primary ITP and 27 with primary APS, who were seen at Keio University Hospital, Tokyo, Japan. Forty-seven healthy volunteers were used as a control. None of the subjects had a current or previous diagnosis of HIT. Plasma samples from two patients diagnosed with HIT were used as a positive control for the anti-PF4 antibody assays. All patients with SLE fulfilled the ACR preliminary classification criteria [10]. Primary ITP was defined as isolated thrombocytopenia (platelet count $<100 \times 10^9$/l) with a normal peripheral blood smear and no underlying conditions that could account for the thrombocytopenic state [11]. Patients meeting the revised Sapporo criteria were categorized as APS [12], and APS patients without underlying immunological diseases such as SLE were categorized as having primary APS. All samples were collected after patients and control subjects gave informed, written consent according to the Declaration of Helsinki. This study has been approved by the Keio University Institutional Review Board.

Clinical features of SLE patients

Demographic and clinical information was obtained from all SLE patients by retrospective chart review at the time blood samples were collected. Thirty-seven clinical and laboratory findings observed during the entire disease course were recorded; these were individual items included in the ACR preliminary classification criteria [10] and the SLEDAI [13], as well as any history of thromboembolism, fetal loss or previous heparin exposure. The SLEDAI was calculated at the time of blood sampling.

Autoantibody assays

IgG aCL and IgM isotypes were assayed by a commercial ELISA kit (MBL, Nagoya, Japan). LA was determined by a cross-mixing test using a commercially available kit based on the dilute Russell's viper venom test (Gradipore, Sydney, Australia). Anti-dsDNA antibody levels were measured by a commercial ELISA kit (MBL). Antibody response to GPIIb/IIIa, a major platelet autoantigen recognized by anti-platelet antibodies in ITP patients, was evaluated by an enzyme-linked immunospot assay for circulating B cells producing IgG anti-GPIIb/IIIa antibodies [14].

Detection of heparin-dependent anti-PF4 antibodies

Heparin-dependent anti-PF4 antibodies were measured using an ELISA kit for anti-PF4–heparin antibodies (GTI, Waukesha, WI, USA) with a microtitre plate coated with a complex of PF4 and polyvinylsulphonate, and anti-IgG/A/M secondary antibodies, according to the manufacturer's protocol. Polyvinylsulphonate is an anionic polymer that provides a high density of negative charges and mimics unfractionated heparin. Samples were tested in duplicate. Antibody units were calculated from optical density read at 450 nm (OD_{450}) using a standard curve obtained from serial concentrations of an HIT serum containing a high titre of heparin-dependent anti-PF4 antibodies. The cut-off level represented the mean plus four times the S.D. of 30 healthy individuals (3.3 U). To confirm that antibody binding was heparin-dependent, sera were applied to the PF4–heparin ELISA in the presence or absence of a high concentration of unfractionated heparin (100 U/ml); this disrupts the PF4–heparin complex, such that its antigenicity for heparin-dependent anti-PF4 antibodies is lost [15]. Results were expressed as an inhibition rate (%) calculated according to the following formula: $100 \times (1 - \text{sample } OD_{450} \text{ in the presence of heparin/sample } OD_{450} \text{ in the absence of heparin})$. An inhibition rate exceeding 40% was regarded as significant, based on previous reports [15, 16]. Patients were considered positive for heparin-dependent anti-PF4 antibodies when the results from both the PF4–heparin ELISA and the confirmatory heparin inhibition assay were positive.

Detection of heparin-independent anti-PF4 antibodies

Heparin-independent anti-PF4 antibodies were measured by an ELISA system, that was developed based on a previously described strategy [17]. Briefly, polyvinyl 96-well plates were coated with human PF4 (ARP, Belmont, MA, USA) at 0.5 μg/ml and blocked with 3% BSA. The wells were incubated with plasma samples diluted at 1:50, and subsequently with peroxidase-conjugated goat anti-human IgG/A/M (ICN/Cappel, Aurora, OH, USA). All samples were tested in duplicate. Antibody units were calculated from the OD_{450} using a standard curve obtained from serial concentrations (0.0625 – 4 μg/ml) of rabbit anti-human PF4 polyclonal antibodies (Chemicon International, Temecula, CA, USA) and peroxidase-conjugated goat

anti-rabbit IgG secondary antibodies (ICN/Cappel). One unit of heparin-independent anti-PF4 antibody was defined as 0.625 μg/ml of rabbit anti-human PF4 antibody. The cut-off value was the mean plus four times the S.D. of 30 healthy individuals (7.4 U).

Serotonin-release assay

The functional capacity of heparin-dependent anti-PF4 antibodies to activate platelets *in vitro* was assessed by a serotonin-release assay as described elsewhere [7]. Briefly, platelet-rich plasma from healthy individuals was incubated with [^{14}C]serotonin (0.1 μCi/ml; Amersham Biosciences, Uppsala, Sweden), and then with the patient's heat-inactivated plasma in the presence of unfractionated heparin (0.1 or 100 U/ml). The reaction was stopped by 0.5% EDTA and supernatants were obtained by centrifugation. The radioactivity of the supernatant was quantified by liquid scintigraphy. The release of [^{14}C]serotonin was calculated as the radioactivity in platelet supernatants relative to the total platelet serotonin uptake. Samples were considered positive if the serotonin release induced in the presence of 0.1 U/ml heparin was >20% of the total serotonin uptake, and was decreased by >50% in the presence of 100 U/ml heparin [18].

ELISA competition assay

To evaluate the specificity of heparin-dependent and -independent anti-PF4 antibodies, diluted plasma samples were pretreated with serial concentrations of human PF4 (0, 5, 10, 20 and 40 μg/ml) before being subjected to the ELISA.

Statistical analysis

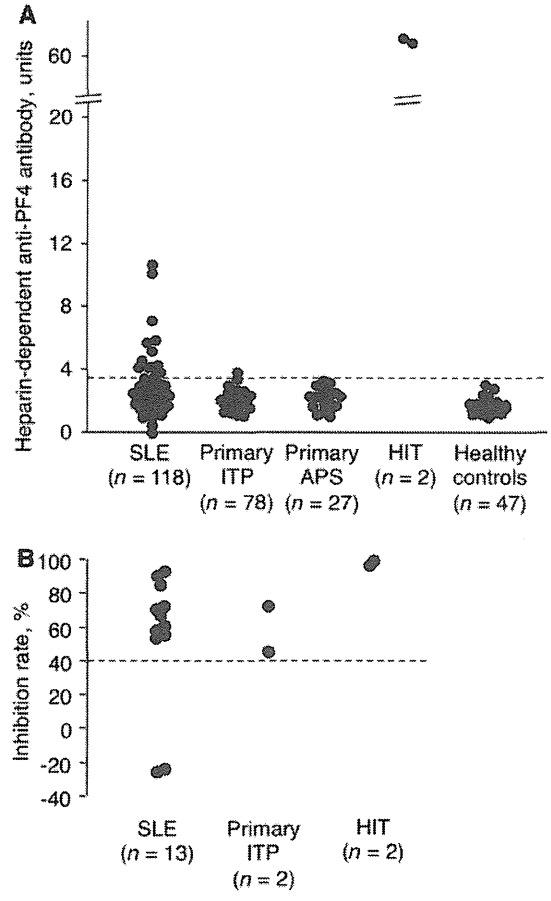
Continuous variables were shown as the mean (S.D.). Differences between two groups were tested for statistical significance using the chi-square test or Fisher's two-tailed exact test where applicable, or the non-parametric Mann–Whitney U-test. The odds ratio (OR) with a 95% CI was calculated for a statistically significant difference. The correlation coefficient was obtained by Spearman's correlation analysis.

Results

Heparin-dependent anti-PF4 antibodies in SLE

We used a PF4–heparin ELISA to screen for heparin-dependent anti-PF4 antibodies in 118 patients with SLE, 78 with primary ITP, 27 with primary APS, 2 with HIT and 47 healthy controls (Fig. 1A). Heparin-dependent anti-PF4 antibodies were detected in 13 patients with SLE (11%) and 2 with primary ITP (3%), but not in any healthy controls or patients with primary APS. Sera from two HIT patients showed remarkably high levels of heparin-dependent anti-PF4 antibodies, suggesting that the antibody titres produced in patients with SLE or primary ITP were low. To confirm that the antibody binding was heparin-dependent, the 17 sera that were positive for heparin-dependent anti-PF4 antibodies by the PF4–heparin ELISA were subjected to a confirmatory

FIG. 1 ELISA detection of heparin-dependent anti-PF4 antibodies.

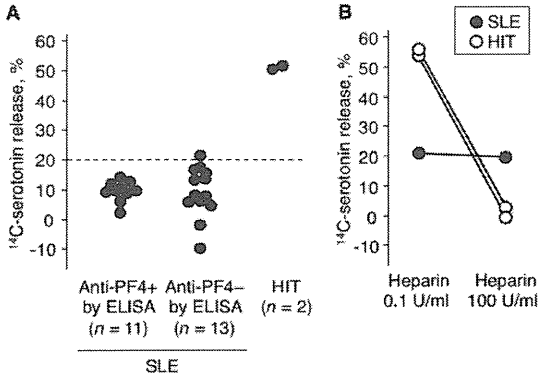


(**A**) Heparin-dependent anti-PF4 antibodies measured by ELISA in sera from 118 patients with SLE, 78 with primary ITP, 27 with primary APS, 2 with HIT and 47 healthy controls. Dotted line: cut-off level for positivity (3.3 U). (**B**) Confirmatory heparin inhibition assay of sera with positive ELISA results for heparin-dependent anti-PF4 antibodies, including sera from 13 patients with SLE, 2 with primary ITP and 2 with HIT. The result was considered positive if the inhibition rate exceeded 40% (dotted line).

heparin inhibition assay (Fig. 1B). In all but two SLE sera, the antibody reactivity was significantly suppressed in the presence of a high heparin concentration. As a result, 11 patients with SLE (9%) were judged to have heparin-dependent anti-PF4 antibodies.

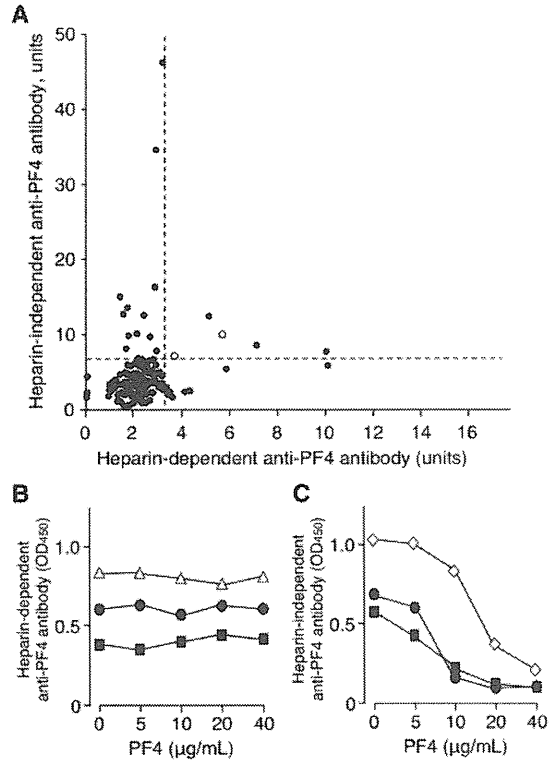
We further evaluated the capacity of the heparin-dependent anti-PF4 antibodies to promote *in vitro* platelet aggregation in the presence of a therapeutic concentration of heparin (0.1 U/ml). The 11 SLE sera positive for heparin-dependent anti-PF4 antibodies were applied to this assay, while 13 randomly selected SLE sera negative

FIG. 2 Serotonin-release assay for functional heparin-dependent anti-PF4 antibodies.



(**A**) A [^{14}C]serotonin-release assay with a low heparin concentration (0.1 U/ml) for SLE patients (11 ELISA-positive and 13 ELISA-negative for heparin-dependent anti-PF4 antibodies) and 2 HIT patients. Dotted line: cut-off (20%). (**B**) [^{14}C]serotonin-release assay with a high heparin concentration (100 U/ml) for three patients with positive serotonin-release assay results in the presence of low heparin concentration, including one with SLE (closed circle) and two with HIT (open circles). Sera that released serotonin in low heparin, and showed a decreased release by >50% in high (100 U/ml) heparin, were considered positive.

FIG. 3 Specificity of two types of anti-PF4 autoantibodies.



(**A**) Correlation between heparin-dependent and -independent anti-PF4 antibody levels in 118 SLE patients. Dotted lines indicate the cut-offs for positivity: 3.3 and 7.4 U for heparin-dependent and -independent anti-PF4 antibodies, respectively. Open circles denote two patients with positive results on the PF4–heparin ELISA, but negative confirmatory heparin inhibition assays. (**B** and **C**) Competitive inhibition in heparin-dependent and -independent anti-PF4 antibody ELISA using PF4 as a competitor. Closed circles and squares denote samples positive for both heparin-dependent and -independent anti-PF4 antibodies. Open triangles and rhombus show a sample positive for heparin-dependent or -independent anti-PF4 antibodies alone, respectively.

for heparin-dependent anti-PF4 antibodies were used as a reference. As shown in Fig. 2A, only one of the ELISA-negative sera had a positive (quite weak) reaction. However, this reactivity was not inhibited by a high heparin concentration (100 U/ml) (Fig. 2B), indicating that none of these SLE sera, including the ones with positive PF4–heparin ELISA results, exerted functional properties in the serotonin-release assay. In contrast, the two HIT sera strongly promoted *in vitro* platelet activation in the presence of a low heparin concentration, and this reactivity was inhibited in the presence of a high heparin concentration.

Correlation between heparin-dependent and -independent anti-PF4 antibodies

ELISA using PF4 antigen in the absence of heparin found heparin-independent anti-PF4 antibodies in 17 (14%) of 118 SLE patients, but in none of the 47 healthy controls. Fig. 3A shows the distribution of heparin-dependent and -independent anti-PF4 antibody levels in the 118 patients with SLE; there was no correlation between the levels of the two antibodies ($r^2 = 0.04$). Twelve (71%) of 17 patients positive for the heparin-independent antibody gave a negative result in ELISA for the heparin-dependent antibody. Only five patients had positive ELISA values for both antibody specificities, but two of them were found to have false-positive PF4–heparin ELISA results [8] because the confirmatory heparin inhibition assay was negative.

To further evaluate the specificity of heparin-dependent and -independent anti-PF4 antibodies, ELISA competition assays were performed using PF4 as a competitor (Fig. 3B and C). Heparin-dependent anti-PF4 antibody reactivity was not inhibited by pretreatment of plasma samples with serial concentrations of human PF4. In contrast, heparin-independent anti-PF4 antibody reactivity was inhibited by PF4 in a dose-dependent manner. This discordant finding was obtained from two patients positive for both heparin-dependent and -independent anti-PF4

antibodies (closed circles and squares). Thus the repertoires of anti-PF4 autoantibodies detected by heparin-dependent and -independent ELISA are essentially separate.

Clinical features of SLE patients with heparin-dependent or -independent anti-PF4 antibodies

Demographic and clinical features were compared between SLE patients with and without heparin-dependent anti-PF4 antibodies (Table 1). Thrombocytopenia was more frequently detected in patients with heparin-dependent anti-PF4 antibody than in those without (46 *vs* 12%; $P = 0.007$; OR = 6.0; 95% CI 1.6–22.6). All five patients with thrombocytopenia had circulating B cells producing IgG anti-GPIIb/IIIa antibodies. There was no difference in the frequency of IgG aCL or LA, but IgM aCL was more common in patients with heparin-dependent anti-PF4 antibody than in those without (64 *vs* 25%; $P = 0.007$; OR = 5.2; 95%CI 1.4–19.1). The frequencies of other clinical and serological features, including thromboembolism and fetal loss, were similar between the two groups. In addition, SLEDAI at blood sampling was comparable. There was no correlation between the heparin-dependent anti-PF4 antibody level and SLEDAI. Interestingly, a previous history of heparin exposure was found solely in a small proportion of patients who did not have heparin-dependent anti-PF4 antibodies.

On the other hand, there was no difference in the clinical and serological features during the entire disease course between SLE patients stratified by the presence or absence of heparin-independent anti-PF4 antibodies. However, SLEDAI at blood sampling was significantly higher in patients with heparin-independent anti-PF4 antibodies than in those without [10.9 (6.1) *vs* 2.5 (3.4); $P < 0.0001$]. Anti-dsDNA antibody levels did not differ between heparin-independent anti-PF4 antibody-positive and -negative groups [80.9 (82.6) *vs* 59.1 (78.7); $P = 0.29$]. Heparin-independent anti-PF4 antibody levels were positively correlated with SLEDAI ($P = 0.0005$, $r^2 = 0.51$) (Fig. 4). Levels of anti-dsDNA antibody, which is known as a marker for disease activity in SLE, were also correlated with SLEDAI ($P = 0.003$, $r^2 = 0.35$). Interestingly, levels of heparin-independent anti-PF4 and anti-dsDNA antibodies were not correlated with each other ($r^2 = 0.05$). Again, none of the patients with heparin-independent anti-PF4 antibodies had previous heparin exposure.

Discussion

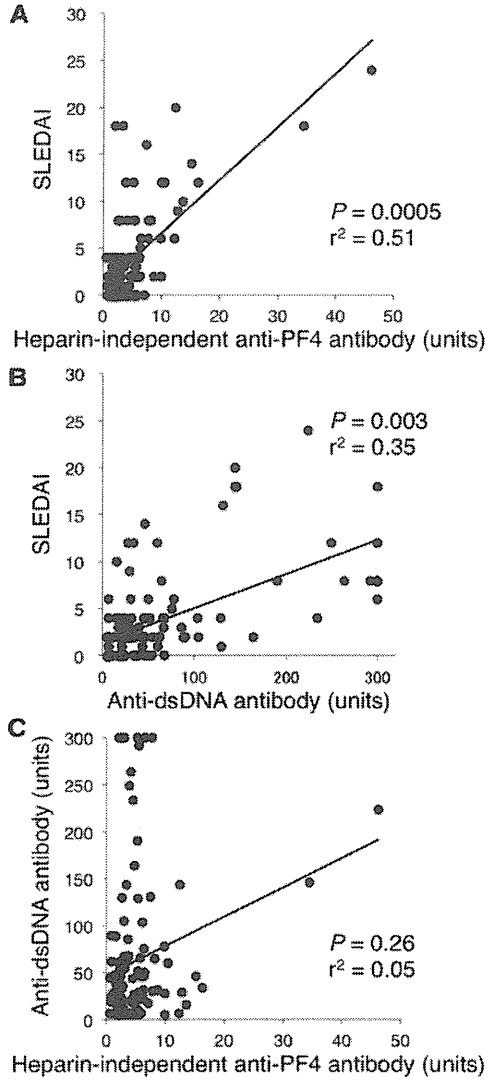
In the present study we found autoantibodies against PF4, either associated with heparin or not, in a significant proportion (~19%) of patients with SLE. Heparin-dependent and -independent anti-PF4 antibody specificities were principally separate. The production of heparin-dependent anti-PF4 antibodies was not linked to previous

TABLE 1 Demographic and clinical features of SLE patients with and without heparin-dependent anti-PF4 antibodies

Clinical characteristics	Heparin-dependent anti-PF4 antibody		P
	Positive ($n = 11$)	Negative ($n = 107$)	
Sex, % female	91	92	0.94
Age at SLE onset, years	38.4 (8.9)	41.8 (13.6)	0.22
Malar rash, %	36	62	0.11
Discoid rash, %	9	9	0.98
Photosensitivity, %	36	36	0.99
Oral ulcers, %	27	26	0.94
Arthritis, %	82	64	0.23
Serositis, %	18	19	0.97
Renal disorder, %	18	38	0.19
Haemolytic anaemia, %	0	8	0.76
Neurological disorder, %	0	4	0.82
Leucopenia, %	64	59	0.76
Lymphopenia, %	55	51	0.84
Thrombocytopenia, %	46	12	0.007
Anti-dsDNA antibody, %	73	82	0.44
Anti-dsDNA antibody level, units	72.1 (113.5)	61.3 (75.6)	0.67
IgG aCL, %	36	45	0.59
IgM aCL, %	64	25	0.007
LA, %	36	36	0.96
Thromboembolism, %	36	20	0.20
Fetal loss, % (n+/n)	14 (1/7)	14 (6/42)	1.00
Secondary APS, %	27	17	0.39
History of heparin exposure, %	0	9	0.62
SLEDAI	3.3 (2.9)	3.7 (5.0)	0.78

Values are represented as the mean (S.D.), unless otherwise indicated.

FIG. 4 Correlations between heparin-independent anti-PF4 antibody levels, anti-dsDNA antibody levels and SLEDAI at blood sampling in 118 patients with SLE.



(**A**) Correlation between heparin-independent anti-PF4 antibody levels and SLEDAI. (**B**) Correlation between anti-dsDNA antibody levels and SLEDAI. (**C**) Correlation between heparin-independent anti-PF4 antibody and anti-dsDNA antibody levels.

heparin exposure or to typical clinical HIT features, but was associated with thrombocytopenia and IgM aCL. On the other hand, heparin-independent anti-PF4 antibody levels were correlated with lupus activity measured by SLEDAI.

In our assay systems, repertoires of heparin-dependent and -independent anti-PF4 antibodies appeared to be separate, since (i) levels of these two antibodies did not correlate at all; and (ii) ELISA competition assay indicated no cross-reactivity between free PF4 and PF4 bound to anionic polymer surfaces. It has been reported that heparin-dependent anti-PF4 antibodies detected in patients with HIT recognize ultra-large complexes of PF4 and heparin, but do not react with PF4 in its free form [19]. PF4 binds with high affinity to the negatively charged surfaces, resulting in a dramatic change in its conformation [20]. The precise mechanisms for lack of heparin-independent anti-PF4 antibody reactivity in the PF4–heparin ELISA remain unclear, but the epitope(s) recognized by heparin-independent anti-PF4 antibodies may be masked by competition with heparin for the binding site and/or lost by the conformational change of PF4.

The relatively low prevalence of heparin-dependent anti-PF4 antibodies in our cohort of SLE patients (9%) was nearly concordant with previous studies, in which the prevalence was 4–15% [5, 6]. In the majority of sera with positive PF4–heparin ELISA results, the antibody reactivity was inhibited in the presence of a high heparin concentration, indicating the heparin-dependent nature of the antibody binding. However, none of these sera tested positive by the serotonin-release assay, a gold standard functional assay for diagnosing HIT. This was probably due to the relatively low antibody titres found in patients with SLE, since the ELISA system is more sensitive than the serotonin-release assay [21]. Interestingly, the incidence of heparin-dependent anti-PF4 antibodies is surprisingly high in patients exposed to heparin, exceeding a quarter of all exposed patients, although only a small portion of these patients develop HIT [22]. Antibody titre has been shown to be one of the factors determining whether individuals with heparin-dependent anti-PF4 antibodies remain asymptomatic or develop HIT [23]. Alternatively, the apparent discrepancy between the ELISA and serotonin-release assays might be explained by a lack of heparin-dependent anti-PF4 antibodies of the IgG isotype in SLE sera, since *in vitro* platelet activation is mediated through IgG antibodies that bind to the heparin–PF4 complex and cross-link with FcγRIIA receptors expressed on platelet surfaces [24]. In this regard, it has been reported that the predominant isotype found in heparin-dependent anti-PF4 antibodies in SLE patients is IgM [7], which does not interact with the FcγRIIA receptor.

In our cohort, the only clinical feature associated with the presence of heparin-dependent anti-PF4 antibodies was thrombocytopenia, although this association has not been described in previous studies [6, 7]. The presence of the anti-GPIIb/IIIa autoantibody response we observed in thrombocytopenic patients with heparin-dependent anti-PF4 antibodies strongly suggests that the thrombocytopenia is caused mainly by anti-platelet autoantibodies to GPIIb/IIIa that accelerate platelet clearance through the reticuloendothelial system (the pathogenic process in ITP) rather than by the consumption of platelets through their activation and the acceleration of the coagulation pathway (the pathogenic process in HIT). Since the PF4 protein is stored abundantly in platelet granules, it is possible that heparin-dependent anti-PF4

antibodies are produced simply as a consequence of excessive platelet destruction. The presence of heparin-dependent anti-PF4 antibodies in some patients with primary ITP supports this hypothesis.

In contrast, the presence of heparin-independent anti-PF4 antibodies was associated with SLE disease activity. Interestingly, there was no correlation between levels of heparin-independent anti-PF4 and anti-dsDNA antibodies, suggesting that measurement of heparin-independent anti-PF4 antibodies together with anti-dsDNA antibodies may provide additional information useful in evaluating disease activity in SLE patients. A circulating level of PF4 is maintained in a very low level (<20 ng/ml), because PF4 released by activated platelets rapidly disappears from circulation by binding to endogenous heparinoids expressed on endothelial cells [25]. In patients with active SLE, vascular inflammation and resultant endothelial injury in affected tissues, such as kidney and central nervous system, may lead to local release of an excessive amount of PF4 from activated platelets and damaged endothelium. In this case, circulating heparin-independent anti-PF4 autoantibodies may accelerate vascular damage by forming immune complexes by binding to free PF4.

In contrast to a previous report by Alpert *et al.* [7], we did not observe an association between heparin-dependent anti-PF4 antibodies and thromboembolism or an APS diagnosis. In addition, heparin-dependent anti-PF4 antibodies were not detected in any patients with primary APS. The discrepancy in these results may be due to the small number of APS patients enrolled in our study. On the other hand, our study reproduced a significant correlation between heparin-dependent anti-PF4 antibodies and IgM aCL reported by Alpert *et al.* A partial cross-reactivity between heparin-dependent IgM anti-PF4 antibody and IgM aCL may be responsible, in part, for this relationship [7, 26].

None of the SLE patients with heparin-dependent anti-PF4 antibodies had developed clinical HIT at the time of this study, but the presence of this autoantibody may be clinically important. In individuals with a history of HIT and low-titre heparin-dependent anti-PF4 antibodies, HIT frequently recurs upon re-exposure to heparin [27]. In addition, preexisting heparin-dependent anti-PF4 antibodies without previous heparin exposure increases the risk of developing HIT following an invasive procedure requiring heparin use [28, 29]. Taken together, it is likely that SLE patients with low-titre heparin-dependent anti-PF4 antibodies may also have an increased risk for developing HIT when treated with heparin. In this regard, a recent retrospective cohort study of 25 653 patients receiving heparin products found a strong correlation between autoimmune diseases, such as SLE, and the development of HIT [30]. Thus SLE patients with heparin-dependent anti-PF4 autoantibodies should be watched closely for signs of developing HIT, when they are given heparin products.

The mechanisms by which anti-PF4 autoantibodies are produced without heparin exposure in SLE patients remain unclear. Since anti-PF4 antibodies in SLE sera apparently recognize multiple epitopes formed on the PF4–heparin complex as well as on PF4 alone, PF4 itself appears to be a preferred target of the autoimmune response. In this regard, three patients who suffered acute-onset arterial thrombosis and thrombocytopenia with heparin-dependent anti-PF4 autoantibodies despite a lack of previous heparin exposure have been reported [31], suggesting that HIT can present as an autoimmune disease. Given that the presence of autoimmune and inflammatory conditions is a risk factor for developing HIT [30], the vascular inflammation observed in SLE patients may trigger an autoantibody response to PF4 through the release of large amounts of PF4 from platelets, followed by the formation of the antigenic complex with endogenous heparinoids [32]. In addition, a predisposition to autoimmunity in SLE patients may also promote an autoantibody response.

In summary, PF4 is an autoimmune target in patients with SLE. Interestingly, heparin-dependent and -independent anti-PF4 autoantibodies may be involved in different aspects of pathophysiology of SLE. Further prospective studies are needed to evaluate whether heparin-dependent anti-PF4 antibodies preexisting in SLE patients increase their risk of developing HIT when exposed to heparin and whether heparin-independent anti-PF4 antibodies are useful as a biomarker for lupus activity.

Rheumatology key messages

- Low heparin-dependent anti-PF4 autoantibody titres are present in some SLE patients.
- Heparin-dependent anti-PF4 antibodies are associated with thrombocytopenia and IgM aCLs.
- Heparin-independent anti-PF4 antibody levels are correlated with lupus activity.

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Original article

Clinical characteristics and survival of Japanese patients with connective tissue disease and pulmonary arterial hypertension: a single-centre cohort

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Abstract

Objective. To clarify the characteristics, survival and predictors of mortality in Japanese patients with pulmonary arterial hypertension (PAH) associated with CTD.

Methods. This single-centre cohort study enrolled 70 consecutive patients with PAH-CTD who visited a tertiary referral centre in Japan between 1970 and 1990 ($n = 30$, historical group) and between 2000 and 2009 ($n = 40$, recent group). Baseline clinical features, haemodynamic parameters and ANA profiles were recorded. The Cox proportional hazards regression model was used to determine independent factors associated with an increased risk of mortality.

Results. MCTD and SLE were the major underlying CTDs, comprising 43% and 29% of PAH patients respectively, whereas SSc was less common (19%). Anti-U1RNP antibody was the most prevalent ANA (61%). The cumulative survival rate was significantly better in the recent group in comparison with the historical group (76% *vs* 26% at 3 years; $P < 0.001$). When both groups were combined, World Health Organization functional class III or IV at diagnosis was identified as an independent predictor of mortality, whereas modern PAH drug use was associated with a favourable outcome.

Conclusion. The major PAH-CTD population in Japan suffers from MCTD or SLE with anti-U1RNP antibody, in contrast to PAH-CTD patients in the USA and Europe. Modern PAH treatment has improved survival rates, but long-term outcomes are still unsatisfactory. Independent predictors of mortality indicate that early diagnosis and the prompt use of PAH drugs should improve survival.

Key words: pulmonary arterial hypertension, mixed connective tissue disease, systemic lupus erythematosus, scleroderma, anti-U1RNP antibody.

CLINICAL SCIENCE

Introduction

Pulmonary arterial hypertension (PAH) is an intractable condition that in patients with CTDs has progressive debilitating symptoms and a poor prognosis [1–3]. In cohort studies conducted in the USA and Europe, the majority of patients with PAH associated with CTD (PAH-CTD) had SSc [4–7], and the most prevalent serum ANA was an ACA [8–12]. However, in our clinical experience, SLE and MCTD are fairly common in Japanese patients with PAH-CTD. In fact, a multicentre survey conducted in Japan in the late 1980s revealed a high prevalence of MCTD and serum anti-U1RNP antibodies in CTD patients with pulmonary hypertension (PH) [13], although that study did not require right heart catheterization for the diagnosis of PH, and did not exclude patients with PH due to left-sided heart disease, interstitial lung disease (ILD) or chronic thromboembolism. Therefore,

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Japanese patients with PAH-CTD have not been characterized in detail.

The prognosis for patients with PAH-CTD was very poor before 2000, when non-specific vasodilators were the sole treatment option: the 1-year survival rate in patients with SSc and PAH was only 45% [1]. Over the past decade, PAH-specific vasodilative agents, including prostanoids, endothelin receptor antagonists and phosphodiesterase 5 inhibitors, have become available for clinical use. Consequently, several studies that primarily enrolled SSc patients reported improvement in short-term survival: the 1-year survival rate was >80% [4, 5, 10, 12, 14–16]. In Japan, beraprost and i.v. epoprostenol have been available since 1999, and bosentan and sildenafil were introduced to clinical practice in 2005. However, few data are available on the improvement in survival of Japanese patients with PAH-CTD in the modern treatment era.

In this study we evaluated the clinical characteristics and prognosis of patients with PAH-CTD who were diagnosed and monitored at a tertiary PH referral centre in Japan. By comparing databases covering 1970–90 (pre-PAH drug era) and 2000–09 (modern treatment era), we also investigated the impact of PAH drugs on survival.

Materials and methods

Study population

All the incident cases of PAH-CTD consecutively diagnosed at the specialized PH clinic of Keio University Hospital were included in this study: 30 patients were diagnosed with PAH between 1970 and 1990 (historical group); 40 patients were diagnosed between 2000 and 2009 (recent group). Patients in whom PAH was diagnosed between 1991 and 1999 were excluded because complete clinical data were not available. PAH was diagnosed based on the following criteria: (i) a mean pulmonary arterial pressure (mPAP) $\geqslant$25 mmHg at rest by right heart catheterization [17]; (ii) exclusion of left-sided heart disease, determined by pulmonary capillary wedge pressure $\leqslant$15 mmHg by right heart catheterization; (iii) exclusion of advanced ILD, determined by forced vital capacity predicted <70% [18]; and (iv) exclusion of chronic thromboembolism, determined by contrast-enhanced CT scan and ventilation/perfusion scan with or without pulmonary angiography. The study was approved by the Keio University Institutional Review Board, and clinical information was obtained after the patients had given written informed consent.

The clinical diagnoses of SLE and SSc were made according to the ACR preliminary classification criteria [19, 20]. MCTD was diagnosed according to the criteria proposed by Kasukawa *et al.* [21], as having all of the following: RP or swollen fingers/hands, a positive anti-U1RNP antibody, and overlapping features of at least two diseases among SLE, SSc and polymyositis. SS was diagnosed according to the revised criteria proposed by the American–European Consensus Group [22]. SS patients without any apparent CTDs were regarded as having primary SS.

Data collection

A complete medical history, physical examination, laboratory evaluations and right heart catheterization were performed for each patient at the time of PAH diagnosis, with more limited evaluations during follow-up. Except in SSc patients, the onset of CTD was defined as the first symptom attributable to CTD; in SSc patients, disease onset was defined as the first non-RP manifestation. The follow-up clinical and laboratory information was prospectively recorded in the database. Data collected at the time of PAH diagnosis included age, sex, underlying CTD, RP, ILD, pericardial effusion, renal disorder, World Health Organization-functional class (WHO-FC) and haemodynamic parameters. ILD was defined as bibasilar reticular pattern of linear or lineonodular densities most pronounced in basilar portions of the lungs in chest radiographs and/or CT scans [20]. Pericardial effusion was detected by echocardiography. Renal disorder was defined as persistent proteinuria >0.5 g/day and/or the presence of cellular casts [19]. All treatment regimens used for PAH were recorded, and included PAH drugs (beraprost, epoprostenol, bosentan and sildenafil) and immunosuppressive treatment, which consisted of CSs (>0.5 mg/kg prednisolone equivalent) in combination with or without AZA, CYC or MMF.

ANA profiles

A serum sample was obtained from each patient at the time of PAH diagnosis and stored at −20°C. Serum ANA profiles were identified by IIF on HeLa cell chromosomal spreads (anticentromere), radioimmunoassay (anti-double-stranded DNA), RNA immunoprecipitation (anti-U1RNP, anti-Sm, anti-SSA and anti-SSB) and protein immunoprecipitation (anti-topo I) [23].

Statistical analysis

Continuous variables were compared with the Mann–Whitney U-test. Categorical variables were compared with the chi-square test. For a 2×4 contingency table, significant differences (overall $P < 0.05$) were further analysed by pairwise comparisons. Survival analysis was performed using the Kaplan–Meier method, and the survival rates of the two groups were compared using the log-rank test. The Cox proportional hazards regression model was used to determine factors associated with an increased risk of mortality at 1, 3 and 5 years after PAH diagnosis. Variables selected by univariate analysis were further subjected to multivariate analysis. The results are presented as a hazard ratio (HR) with 95% CI. Statistical analysis was performed using SPSS 17.0 statistical software (SPSS; Chicago, IL, USA).

Results

Clinical characteristics

Table 1 shows the baseline characteristics and treatment regimens of 70 patients with PAH-CTD. Almost half of patients were referred because of a suspicion of PAH. In the others, PAH was diagnosed during regular follow-up at a rheumatology clinic. MCTD was the most common underlying CTD (43%), followed by SLE (29%), SSc (19%) and primary SS (10%). Eleven (85%) of the 13 SSc patients with PAH were classified as having lcSSc. The most prevalent ANA was anti-U1RNP antibody (61%). Anti-SSA antibody was detected in 38 patients (54%), including 6 in whom the anti-SSA antibody with or without anti-SSB antibody was the only known ANA specificity. In contrast, the prevalence of ACA was only 16%. At the time of PAH diagnosis, 69% of patients were classified as WHO-FC III or IV, with moderately or severely impaired haemodynamics. At least one PAH drug was administered to 56% of the patients immediately after the diagnosis of PAH, and 41% received a sequential combination therapy consisting of two or three PAH drugs. Immunosuppressive treatment was given to 37% of the patients at the time of PAH diagnosis. Seventeen patients (27%) had radiological evidence of ILD, but all of them presented with minimal disease activity on imaging and normal or slightly depressed restrictive ventilatory function (FVC $\geqslant$70%).

Comparison of the baseline characteristics between the historical and recent groups (Table 1) revealed a greater frequency of referral in recent cases ($P = 0.001$), an older age at PAH diagnosis ($P < 0.001$), a lower frequency of RP ($P < 0.001$) and a higher frequency of ILD ($P = 0.04$). The haemodynamic parameters tended to be worse in recent cases than in historical ones. Finally, as expected, at least one PAH drug was administered to all except one recent patient, whereas none were administered to historical patients ($P < 0.001$).

TABLE 1 Demographic and clinical characteristics at PAH diagnosis and treatment regimens for patients with PAH-CTD

Demographic and clinical features	All patients ($n = 70$)	Historical group ($n = 30$)	Recent group ($n = 40$)	P^a
Referral patients (%)	49	27	65	0.001
Female (%)	100	100	100	1.0
Age at PAH diagnosis (years)	40 (16)	31 (10)	47 (17)	<0.001
Time between CTD onset and PAH diagnosis (months)	74 (89)	35 (32)	103 (106)	0.03
RP (%)	74	97	58	<0.001
ILD (%)	27	13	38	0.04
Pericardial effusion (%)	24	20	28	0.7
Renal disorder (%)	7	3	10	0.4
Underlying CTD (%)				
MCTD	43	63	28	0.08
SLE	29	20	35	
SSc	19	13	23	
Primary SS	10	3	15	
ANA (%)				
Anti-U1RNP	61	70	56	0.3
Anti-Sm	16	13	18	0.9
Anti-SSA	54	67	45	0.07
Anti-SSB	20	23	18	0.8
Anticentromere	16	7	23	0.1
Anti-topo I	1	0	3	1.0
Anti-double-stranded DNA	23	17	28	0.4
WHO-FC (%)				
Class I	3	0	5	0.3
Class II	29	30	28	
Class III	56	47	63	
Class IV	13	23	5	
Haemodynamic parameters				
mPAP, mmHg	45 (12)	42 (14)	47 (10)	0.06
CO, l/min	3.7 (1.1)	4.0 (1.0)	3.6 (1.2)	0.07
PVR, Wood units	11.6 (6.0)	9.9 (5.8)	12.9 (6.0)	0.02
Treatment regimen (%)				
At least one PAH drug	56	0	98	<0.001
Immunosuppressive treatment	37	43	33	0.4

Values are presented as the mean (S.D.), unless otherwise indicated. [a]Comparisons between historical and recent groups.

TABLE 2 Demographic and clinical characteristics at PAH diagnosis in patients with MCTD, SLE, SSc and primary SS

Demographic and clinical features	MCTD ($n=30$)	SLE ($n=20$)	SSc ($n=13$)	Primary SS ($n=7$)	Overall P
Age at PAH diagnosis (years)	37 (12)	32 (12)	56 (13)	50 (20)	<0.001[a]
Time between CTD onset and PAH diagnosis (months)	51 (56)	88 (107)	116 (110)	56 (91)	0.2
RP (%)	97	45	92	29	<0.001[b]
ILD (%)	33	10	31	43	0.4
Pericardial effusion (%)	27	35	15	0	0.6
Renal disorder (%)	0	25	0	0	0.03[c]
ANA (%)					
Anti-U1RNP	100	55	8	14	<0.001[d]
Anti-Sm	27	15	0	0	0.3
Anti-SSA	63	60	8	86	<0.001[e]
Anti-SSB	27	20	0	29	0.4
Anti-centromere	0	10	60	14	<0.001[f]
Anti-topo I	0	0	8	0	0.5
Anti-double-stranded DNA	17	50	8	0	0.03[g]
WHO-FC (%)					
Class I	0	10	0	0	1.0
Class II	30	20	39	29	
Class III	57	60	46	57	
Class IV	13	10	15	14	
Haemodynamic parameters					
mPAP, mmHg	41 (11)	48 (11)	44 (14)	51 (11)	0.08
CO, l/min	3.8 (1.1)	3.9 (1.3)	3.6 (1.1)	3.1 (0.5)	0.5
PVR, Wood units	10.3 (5.8)	12.2 (5.2)	12.0 (7.6)	15.4 (5.2)	0.1

Values are presented as the mean (S.D.), unless otherwise indicated. Significant differences (overall $P < 0.05$) were further analysed by pairwise comparisons. [a]$P < 0.01$ between MCTD and SLE, SSc or primary SS, and between SLE and SSc; $P = 0.04$ between SLE and primary SS. [b]$P < 0.01$ between MCTD and SLE or primary SS, between SLE and SSc, and between SSc and primary SS. [c]$P < 0.01$ between SLE and MCTD. [d]$P < 0.01$ between MCTD and SLE, SSc or primary SS, and between SLE and SSc. [e]$P < 0.01$ between MCTD, SLE or primary SS and SSc. [f]$P < 0.01$ between MCTD or SLE and SSc. [g]$P < 0.05$ between SLE and MCTD, SSc or primary SS.

Clinical features associated with underlying CTDs

Baseline demographic, clinical and haemodynamic parameters were compared according to underlying CTD (Table 2). Among the four CTD groups, patients with SLE were the youngest at PAH diagnosis, and MCTD patients were the next youngest, compared with patients with SSc or primary SS. RP was observed in nearly all patients with MCTD or SSc but in only 45% and 29% of patients with SLE and primary SS, respectively. Renal disorder was exclusively found in patients with SLE at diagnosis of PAH. The distribution of ANAs corresponded well with the individual CTDs. Anti-U1RNP antibody was predominantly found in MCTD and SLE, whereas anti-SSA antibody was detected broadly in non-SSc CTDs. ACA was the predominant ANA in SSc. There were no statistically significant differences in the WHO-FC or haemodynamic parameters among the four groups.

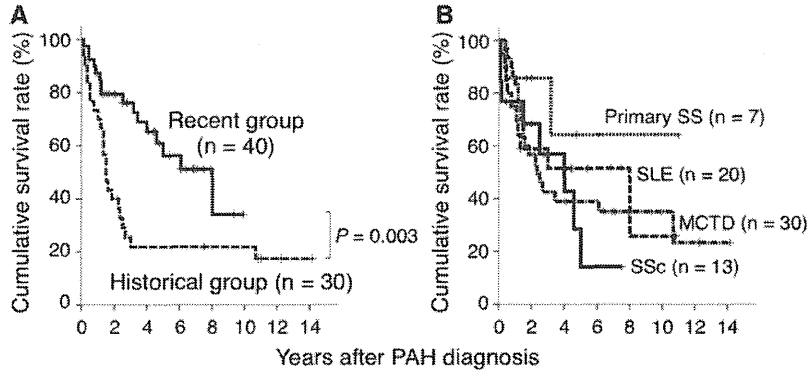
Survival rates

Cumulative survival rate was significantly better in the 40 recent cases than in the 30 historical ones ($P = 0.003$; Fig. 1A), indicating that prognosis has improved in Japanese patients with PAH-CTD. All the deaths were related to PAH, and causes of death included PAH crisis, right heart failure and sudden death probably due to arrhythmia. The 1-, 3- and 5-year survival rates were 73%, 22% and 22%, respectively, in the historical group, and 87%, 76% and 53%, respectively, in the recent group. Differences in the cumulative survival rate among patients with MCTD, SLE, SSc and primary SS were not statistically significant (Fig. 1B) but there was a trend towards a worse survival rate in SSc patients, compared with patients with all the other CTDs combined, in the recent group ($P = 0.08$).

Prognostic factors

To identify variables that predict outcomes at 1, 3 and 5 years after the PAH diagnosis in patients with PAH-CTD, we first performed a univariate analysis to find prognostic factors from the baseline characteristics and treatment regimens. An older age at PAH diagnosis and WHO-FC III/IV were associated with poor survival rates, whereas the use of any PAH drug was positively associated with survival at 1, 3 or 5 years. Additionally, a lower cardiac output (CO) and higher pulmonary vascular resistance (PVR) were associated with poor survival rates at 1 year and ILD with poor survival rates at 3 years. None of the underlying CTDs or ANAs were found to be a prognostic factor.

FIG. 1 Cumulative survival rates in 70 patients with PAH-CTD.



(**A**) The cumulative survival rates of the recent group and the historical group were compared. (**B**) The cumulative survival rates of the two groups were compared according to underlying CTD: MCTD, SLE, SSc or primary SS. Comparisons between the two groups were made using the log-rank test.

TABLE 3 Independent predictors of mortality at 1, 3 and 5 years after PAH diagnosis, obtained by multivariate analysis

	1 year		3 years		5 years	
Selected variables	HR (CI)	*P*	HR (CI)	*P*	HR (CI)	*P*
Age at PAH diagnosis	1.01 (0.96, 1.07)	0.7	0.99 (0.96, 1.04)	0.9	1.00 (0.97, 1.04)	0.9
ILD	1.38 (0.34, 5.61)	0.7	1.25 (0.36, 4.39)	0.7	1.67 (0.58, 4.78)	0.3
WHO-FC III or IV	2.53 (0.24, 26.3)	0.4	8.80 (1.81, 42.8)	0.007	10.83 (2.73, 43.0)	0.001
CO	0.60 (0.15, 2.31)	0.5	0.81 (0.42, 1.60)	0.6	0.68 (0.35, 1.35)	0.3
PVR	1.07 (0.81, 1.39)	0.7	1.07 (0.88, 1.29)	0.5	1.03 (0.87, 1.23)	0.7
Use of any PAH drug	0.11 (0.02, 0.72)	0.02	0.12 (0.03, 0.40)	0.001	0.11 (0.04, 0.35)	<0.001

Variables selected by univariate analysis were then subjected to multivariate analysis to find independent factors that predicted an increased risk of mortality. As shown in Table 3, WHO-FC III/IV was identified as a prognostic factor for mortality at 3 and 5 years ($P = 0.007$ and 0.001, respectively). Additionally, the use of any PAH drug was a factor for favourable outcomes at 1, 3 and 5 years ($P = 0.02$, 0.001 and <0.001, respectively).

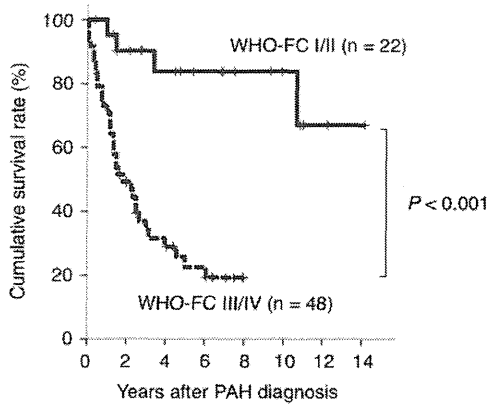
To confirm the impact of the WHO-FC at PAH diagnosis on survival rates, the cumulative survival rate was compared between patients with WHO-FC I/II and those with FC III/IV (Fig. 2). The cumulative survival rate was higher in patients with WHO-FC I/II ($P < 0.001$). The 1- and 3-year survival rates were 95% and 90%, respectively, in patients with WHO-FC I/II, compared with 73% and 37%, respectively, in those with WHO-FC III/IV. We further evaluated the impact of the treatment regimen on the outcome in patients with WHO-FC I/II and III/IV separately (Fig. 3). In patients with WHO-FC III/IV, the cumulative survival rates were significantly different in patients who were and were not treated with PAH drugs, but the difference in patients with WHO-FC I/II was not statistically significant. On the other hand, there was no difference in the survival rates of patients who received and did not receive immunosuppressive treatment, regardless of the WHO-FC.

Discussion

This study, conducted at a single specialized PH centre, is the largest cohort study of Japanese patients with PAH-CTD. We found that the characteristics of Japanese patients with PAH-CTD included high frequencies of MCTD and SLE as underlying CTD, and a high frequency of serum anti-U1RNP antibody. These features are in sharp contrast to cohort studies conducted in the USA and Europe, in which SSc patients were found to comprise >70% of the patients with PAH-CTD [4–7], and ACA was the most frequent ANA [8-12, 24]. It is possible that MCTD patients were categorized as having SSc in the USA and European cohorts, since whether MCTD is a distinct disease has been a matter of controversy [25]. However, the anti-U1RNP antibody was infrequent in patients with SSc-associated PAH in the studies conducted in the USA [8, 24].

The mean age at PAH diagnosis in Japan was 40, which was younger than the 49–56 year range reported for the US and European cohorts [4–7]. This difference might be explained by the higher frequencies of MCTD and SLE

FIG. 2 Cumulative survival rates in 70 patients with PAH-CTD, stratified by WHO-FC.



A comparison between groups was made using the log-rank test.

among the Japanese patients with PAH-CTD. Only a minor group of Japanese patients with PAH-CTD had SSc but these patients showed lcSSc, positive ACA and a relatively long disease duration at PAH diagnosis, which were all consistent with features reported in the USA and European patients with SSc-associated PAH [8, 26]. The distribution of underlying CTDs in our study was principally concordant with a recent study from northern Japan, which described a cohort of patients with PAH-CTD containing eight with SLE, four with MCTD, four with primary SS, three with SSc and one each with RA and DM [27]. Interestingly, reports from eastern Asian countries, including Taiwan and China, show a higher frequency of SLE than SSc as an underlying CTD for PAH [28, 29]. Additionally, PAH was reported as the third leading cause of death in Korean patients with SLE [30]. The reasons for these ethnic differences in the distribution of underlying CTDs in patients with PAH-CTD remain unclear but genetic and environmental factors may play a role.

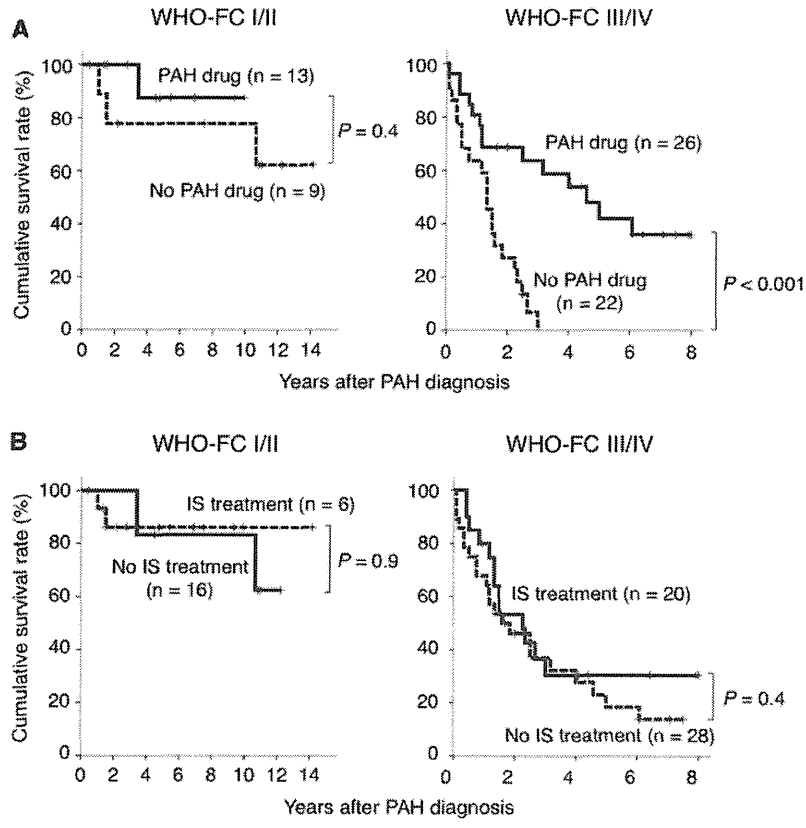
It has been proposed that early detection and therapeutic intervention are important for improving the survival rates of patients with PAH [31]. In support of this idea, we identified WHO-FC III/IV at PAH diagnosis as an independent risk factor for mortality, as reported previously [5, 15, 24], although it is a subjective indicator. We did not observe an improvement in short-term outcomes in WHO-FC I/II patients treated with PAH drugs but this may have been due to the small number of patients. To achieve early detection within the better functional classes, it is essential to screen specific populations that are at high risk of PAH. Patients with SSc, especially lcSSc with long-standing RP, represent a high-risk group, with a 5–10% lifetime risk of developing symptomatic PAH [32]. Therefore, annual screening with echocardiography is recommended for such patients [33], although it has not been demonstrated yet as to which strategy would be cost effective. The same strategy might not be effective in Japan because SSc patients were not the only high-risk population. Moreover, 26% of patients with PAH-CTD did not have RP. Therefore, screening should be expanded to a broad panel of CTDs, including SLE and MCTD, although it is impractical to screen a large number of patients in clinical settings. Given this situation, the anti-U1RNP antibody is a potential hallmark for selecting the high-risk group. Further prospective studies are necessary to identify risk factors that predict the development of PAH in eastern Asian CTD patients, including Japanese, Chinese and Korean patients.

Survival rates were significantly better in recent cases compared with historical ones in our cohort, although baseline haemodynamics tended to be worse in the recent group. Additionally, the use of any PAH drug was an independent predictor for better survival rates. These results indicate that the prognosis of Japanese patients with PAH-CTD has improved in the modern treatment era. However, long-term survival rates were still disappointedly low even in the recent group: the 5-year survival rate was 53%. There are several possible reasons for this poor outcome. First, ~70% of patients already had advanced PAH with WHO-FC III/IV at their diagnosis. Moreover, all patients diagnosed before 2005 received monotherapy with either beraprost or epoprostenol as the first-line therapy. However, similar or even worse outcomes have been reported in recent cohorts of patients with SSc-associated PAH: the 3-year survival rate was 64% in the USA [12], 56% in France [15], 47% in the United Kingdom [5] and 39% in Sweden [11]. The prognosis of PAH is worse in patients with SSc than in those with non-SSc CTDs [5, 16] but the 3-year survival rate in a Chinese cohort that included significant proportions of patients with SLE or MCTD was as low as 54% [29]. Together, these findings indicate that patients with PAH-CTD have a poor prognosis, even in the era of modern PAH-CTD management.

Immunosuppressive treatment is effective for improving symptoms and haemodynamic parameters in some patients with PAH-CTD, especially in those with active-phase SLE or MCTD [27, 34, 35]. Despite these short-term effects, immunosuppressive treatment did not improve long-term survival in our cohort, which contained a high proportion of patients with MCTD and SLE. Therefore, it is likely that the effects of immunosuppressive treatment are limited and PAH drugs should be used in combination with immunosuppressive treatment for patients with PAH associated with MCTD or SLE.

Although this study provides important observations in terms of Japanese patients with PAH-CTD, referral bias should be considered as a potential limitation, since our hospital is the oldest specialized PH centre in the Tokyo metropolitan area. Nearly half of the patients in the present study were referred mainly by cardiologists or pulmonologists and had never received PAH screening. Therefore, the cases most likely to be referred were those with severe PAH and mild CTD-related symptoms. Detection of patients with mild or early PAH would be

FIG. 3 Cumulative survival rates in 70 patients with PAH-CTD, stratified by treatment regimen.



Comparisons were made in patients with WHO-FC I/II and those with WHO-FC III/IV separately. (**A**) The cumulative survival rate was compared between patients who received at least one PAH drug and those who did not. (**B**) The cumulative survival rate was compared between patients who received immunosuppressive (IS) treatment and those who did not. Comparisons between two groups were made using the log-rank test.

expected by a recent campaign aimed to promote the PAH screening of asymptomatic patients with CTD but the recent group included more referral patients than the historical group. This may explain the differences in the baseline characteristics of the historical and recent groups: the haemodynamics were more severe and the frequency of MCTD lower in the recent group. Additionally, as in studies carried out in other east Asian countries [27–30], a small number of the patients in our cohort may have been subject to selection bias. Therefore, the subjects in our study might not reflect the composition of the general PAH-CTD patient population in Japan. Another limitation of this study is the lack of male patients in the study population, since a recent study demonstrated potential differences in baseline haemodynamic characteristics and outcomes between men and women with PAH [36].

In summary, the underlying CTDs and ANA profiles in Japanese patients with PAH-CTD were apparently different from those in the USA and Europe. Modern PAH treatment improves survival rates, but long-term outcomes are still unsatisfactory. Early detection of PAH is important for further improving survival rates but a screening strategy specific to Japanese CTD patients needs to be developed.

Rheumatology key messages

- High frequencies of MCTD/SLE and anti-U1RNP antibody are hallmarks of PAH-CTD in Japanese patients.
- The prognosis of Japanese patients with PAH-CTD has improved with modern treatment.
- WHO-FC at baseline is an independent prognostic factor in Japanese patients with PAH-CTD.

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BRIEF REPORT

Association of HLA–DRB1*0101/*0405 With Susceptibility to Anti–Melanoma Differentiation–Associated Gene 5 Antibody–Positive Dermatomyositis in the Japanese Population

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Objective. **The complication of interstitial lung disease (ILD) in polymyositis/dermatomyositis (PM/DM) is associated with anti–aminoacyl–transfer RNA synthetase (anti-aaRS) antibody or anti–melanoma differentiation–associated gene 5 (anti–MDA-5) antibody positivity. Anti–MDA-5 antibody is associated with clinically amyopathic DM and fatal outcome due to rapidly progressive ILD in Asian populations. The association between genetic factors and anti–MDA-5 antibody–positive DM is unclear. This study was undertaken to investigate the HLA–DRB1 genotype in patients with anti–MDA-5 antibody–positive DM.**

Methods. **We examined genetic differences among 17 patients with anti–MDA-5 antibody–positive DM, 33 patients with anti-aaRS antibody–positive PM/DM, 33 patients with PM/DM without anti-aaRS antibody or ILD, and 265 healthy controls.**

Results. **The frequencies of HLA–DRB1*0101 and DRB1*0405 were 29% and 71%, respectively, in patients with anti–MDA-5 antibody–positive DM, which were higher than the frequencies in healthy controls (10% and 25%, respectively). Among the 17 patients with anti–MDA-5 antibody–positive DM, 16 (94%) harbored either the DRB1*0101 or DRB1*0405 allele. The combined frequency of the DRB1*0101 allele and the DRB1*0405 allele was significantly higher in patients with anti–MDA-5 antibody–positive DM than in patients with PM/DM without anti-aaRS antibody or ILD, with an odds ratio (OR) of 42.7 (95% confidence interval [95% CI] 4.9–370.2) ($P = 1.1 \times 10^{-5}$), or in patients with anti-aaRS antibody–positive PM/DM (OR 13.3 [95% CI 1.6–112.6], $P = 4.5 \times 10^{-3}$).**

Conclusion. **Our findings indicate that HLA–DRB1*0101/*0405 is associated with susceptibility to anti–MDA-5 antibody–positive DM in the Japanese population.**

Dermatomyositis (DM) is characterized by inflammation of the skin and muscle (1) and is occasionally complicated by interstitial lung disease (ILD). In particular, rapidly progressive ILD is an intractable and life-threatening complication. Clinically amyopathic DM (CADM) includes typical skin lesions with amyopathy or hypomyopathy (2). It has recently been reported that patients with CADM who are positive for the anti–melanoma differentiation–associated gene 5 (MDA-5) antibody frequently have complications with rapidly progressive ILD, especially in the Japanese population (3–5). In general, anti–MDA-5 antibody is specific for rapidly progressive ILD associated with CADM and is not detected in patients with CADM or DM without ILD or in patients with polymyositis (PM). The MDA-5 protein plays a role in the innate immune system. MDA-5 initially recognizes picornaviruses, such as coxsackievirus, and induces antiviral responses by producing type I interferons and tumor necrosis factor α (6). Hyperferritinemia is complicated by rapidly progressive ILD in anti–MDA-5 antibody–positive DM (4,5). Although the pathogenesis of rapidly progressive ILD associated with anti–MDA-5 antibody–positive DM has been tentatively attributed to a cytokine storm triggered

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Dr. Kuwana holds a patent on an anti–MDA-5 antibody measuring kit.

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by viral infection, especially in the skin and lungs, its exact mechanism is unknown.

In PM/DM, complication with ILD is associated with the anti–aminoacyl–transfer RNA synthetase (anti-aaRS) antibody or anti–MDA-5 antibody. It has been reported that 90% of Caucasian patients with the anti-aaRS antibody are carriers of HLA–DRB1*03 (7). In the Japanese population, HLA–DRB1*0405 is associated with susceptibility to anti-aaRS antibody–positive PM/DM (8). However, associations between genetic factors and anti–MDA-5 antibody–positive DM have remained unclear.

Therefore, we investigated the HLA–DRB1 gene in patients with anti–MDA-5 antibody–positive DM. In addition, we compared genetic differences in HLA among patients with anti–MDA-5 antibody–positive DM, patients with anti-aaRS antibody–positive PM/DM, and patients with PM/DM without anti-aaRS antibody or ILD.

Table 1. Clinical characteristics and HLA–DRB1 genotype of the patients with anti–MDA-5 antibody–positive DM*

Patient/age/sex	Genotype	Phenotype	ILD type
1/48/M	DRB1*0101/1602	CADM	Rapidly progressive
2/25/F	DRB1*0101/1501	CADM	Chronic
3/53/F	DRB1*0101/0803	CADM	Rapidly progressive
4/18/M	DRB1*0101/1502	CADM	Rapidly progressive
5/47/F	DRB1*0101/0405	DM	Rapidly progressive
6/58/M	DRB1*0405/1406	CADM	Rapidly progressive
7/16/F	DRB1*0405/0401	CADM	Rapidly progressive
8/53/F	DRB1*0405/1501	CADM	Rapidly progressive
9/53/F	DRB1*0405/0410	CADM	Rapidly progressive
10/44/F	DRB1*0405/1406	CADM	Chronic
11/45/F	DRB1*0405/1202	CADM	Chronic
12/39/M	DRB1*0405/0401	CADM	Chronic
13/47/F	DRB1*0405/1201	CADM	Chronic
14/76/F	DRB1*0405/0802	CADM	Rapidly progressive
15/56/F	DRB1*0405/1502	CADM	Rapidly progressive
16/43/M	DRB1*0405/0901	CADM	Chronic
17/66/F	DRB1*0901/1502	CADM	Rapidly progressive

* Anti–MDA-5 = anti–melanoma differentiation–associated gene 5; DM = dermatomyositis; ILD = interstitial lung disease; CADM = clinically amyopathic DM.

PATIENTS AND METHODS

Patients. This retrospective study included patients admitted to Tokyo Women's Medical University Aoyama Hospital or Keio University Hospital from August 1992 to February 2010. Medical records were obtained for 142 and 57 patients diagnosed as having DM and CADM, respectively. The anti–MDA-5 antibody was detected in 31 patients. DNA samples were available for 17 patients with the anti–MDA-5 antibody, and all of these patients were enrolled in the study. All of the enrolled patients had skin rashes, myopathy, or respiratory symptoms (or a combination thereof) at admission. The patients were diagnosed as having DM or CADM based on the criteria of Bohan and Peter (9) or Sontheimer (10), respectively. Specific rashes, including heliotrope rash, Gottron's sign, or Gottron's papules, were used to define DM or CADM. In general, CADM patients present with typical skin lesions and amyopathy or hypomyopathy with a duration of >6 months. A subset of the CADM group included patients who developed fatal ILD within the first 6 months of this study. Clinical data were obtained from hospital admission records.

To investigate the characteristics of the HLA–DRB1 genotype in anti–MDA-5 antibody–positive DM, HLA data were obtained in patients with anti-aaRS antibody–positive PM/DM, patients without anti-aaRS antibody or ILD, and healthy controls. These HLA genotype databases have been described previously (8). All of the subjects in the present study were Japanese. None of the subjects had rheumatoid arthritis (RA) or other connective tissue diseases. This study was approved by the ethics committee of Tokyo Women's Medical University and was performed in accordance with the Declaration of Helsinki.

Evaluation of autoantibodies. Anti–MDA-5 antibody was detected by immunoprecipitation (IP) assay and enzyme-linked immunosorbent assay using recombinant MDA-5 as an antigen, as previously described (3). Anti-aaRS antibodies, including Jo-1, EJ, PL-7, PL-12, and OJ; anti–signal recognition particle (anti-SRP) antibody; anti-Ku antibody; and anti–U1 small nuclear RNP (anti-U1 snRNP) antibody were assessed by RNA IP assays.

Classification of ILD. Patients were evaluated for ILD by chest radiography and computed tomography (CT) or high-resolution CT of the chest. Rapidly progressive ILD was defined as a progressive ILD within 3 months of the onset of respiratory symptoms. Chronic ILD was defined as ILD that was asymptomatic and non–rapidly progressive or slowly progressive over 3 months (11).

HLA–DRB1 genotyping. HLA–DRB1 genotyping was performed using polymerase chain reaction–reverse sequence-specific oligonucleotide techniques and standard methods. The DNA for the HLA–DRB1 genotyping of the patients was extracted from peripheral blood mononuclear cells using standard methods.

Statistical analysis. The chi-square test was used for the comparison of frequencies, and Fisher's exact test was used when appropriate. Data were analyzed using JMP software (SAS Institute). *P* values were adjusted by Bonferroni correction when appropriate.

RESULTS

Clinical characteristics and HLA–DRB1 genotype of patients with anti–MDA-5 antibody–positive DM. As shown in Table 1, 17 patients with anti–MDA-5 antibody–positive DM were enrolled in the study. Their mean ± SD age was 46 ± 16 years. Seventy-one percent were women. The HLA–DRB1*0101 and DRB1*0405 alleles were identified in 5 patients (29%) and 12 patients (71%), respectively. The HLA–DRB1*0101 or *0405 allele was identified in 16 (94%) of the 17

Table 2. Comparison of HLA–DRB1 genotypes among patients with anti–MDA-5 antibody–positive DM, patients with anti-aaRS antibody–positive PM/DM, and patients with PM/DM without anti-aaRS antibody or ILD*

Genotype	Patients with anti–MDA-5 antibody–positive DM (n = 17)	Patients with anti-aaRS antibody–positive PM/DM		Patients with PM/DM without anti-aaRS antibody or ILD		Healthy controls (n = 265)
		PM/DM (n = 33)	DM (n = 19)	PM/DM (n = 33)	DM (n = 21)	
DRB1*0101	29	12	11	12	14	10
DRB1*0401	12	0	0	3	5	2
DRB1*0403	0	9	5	6	5	5
DRB1*0405	71†	42	53	18	24	25
DRB1*0406	0	6	5	3	5	7
DRB1*0407	0	0	0	0	0	2
DRB1*0410	6	3	5	9	5	2
DRB1*0802	6	18	21	9	10	7
DRB1*0803	6	24	21	27	19	14
DRB1*0901	12	24	21	18	19	30
DRB1*1101	0	3	0	0	0	2
DRB1*1201	6	9	16	3	5	7
DRB1*1202	6	6	0	0	0	4
DRB1*1301	0	0	0	3	0	0
DRB1*1302	0	6	5	21	29	19
DRB1*1401	0	3	5	9	10	5
DRB1*1403	0	3	0	6	10	4
DRB1*1405	0	3	5	3	5	6
DRB1*1406	12	0	0	3	5	3
DRB1*1501	12	12	16	9	5	11
DRB1*1502	18	6	11	21	24	20
DRB1*1602	6	0	0	6	0	3
Other	0	0	0	0	0	5

* Values are the percent of subjects. Anti-aaRS = anti–aminoacyl–transfer RNA synthetase; PM = polymyositis (see Table 1 for other definitions).
† $P = 0.0003$ versus patients with PM/DM without anti-aaRS antibody or ILD; $P = 0.00018$ versus healthy controls.

patients. No patients had homoalleles of HLA–DRB1*0101 or DRB1*0405. One patient had both DRB1*0101 and DRB1*0405. With respect to the clinical phenotype, 16 patients had CADM. ILD complication was observed in all of the patients. Moreover, the frequency of rapidly progressive ILD was high (65%). No patients had RA or other connective tissue diseases as complications.

Comparison of the HLA–DRB1 genotype in patients with anti–MDA-5 antibody–positive DM, patients with anti-aaRS antibody–positive PM/DM, and patients with PM/DM without anti-aaRS antibody or ILD. To investigate the characteristics of the HLA–DRB1 genotype in anti–MDA-5 antibody–positive DM, the frequency of the HLA–DRB1 genotype was compared among patients with anti–MDA-5 antibody–positive DM, patients with anti-aaRS antibody–positive PM/DM, patients with PM/DM without anti-aaRS antibody or ILD, and healthy controls (Table 2).

Data previously obtained at our institution indicated that 33 PM/DM patients (14 patients with PM and 19 with DM) exhibited anti-aaRS antibody, as follows: 8 PM patients and 8 DM patients had anti–Jo-1; 4 PM patients and 6 DM patients had anti-EJ; 2 PM patients and 2 DM patients had anti–PL-7; 0 PM patients and 3 DM patients had anti–PL-12; and none of the patients had anti-OJ. Of the 33 patients with anti-aaRS antibody–positive PM/DM, 24 (73%) had ILD. Moreover, 33 PM/DM patients (12 PM patients and 21 DM patients) had neither anti-aaRS antibody nor ILD, and in all 21 of these DM patients, the clinical phenotype was classic DM, not CADM. In patients with PM/DM without anti-aaRS antibody or ILD, anti-SRP antibody, anti-U1 snRNP antibody, and anti-Ku antibody were detected in 3 PM patients, 1 DM patient, and 0 patients, respectively.

As shown in Table 2, the frequency of HLA–DRB1*0101 was ~30% in anti–MDA-5 antibody–positive DM and ~10% in the other subsets, although the difference was not significant ($P = 0.012$ versus

Table 3. Frequency of the HLA–DRB1*0101/0405 alleles in patients with anti–MDA-5 antibody–positive DM*

	Patients with anti–MDA-5 antibody–positive DM (n = 17)	Patients with anti-aaRS antibody–positive PM/DM		Patients with PM/DM without anti-aaRS antibody or ILD	
		PM/DM (n = 33)	DM (n = 19)	PM/DM (n = 33)	DM (n = 21)
DRB1*0101 or DRB1*0405, %	94	55	63	27	33
P†	–	4.5×10^{-3}	4.4×10^{-2}	1.1×10^{-5}	2.0×10^{-4}
OR (95% CI)	–	13.3 (1.6–112.6)	9.3 (1.0–86.4)	42.7 (4.9–370.2)	32 (3.5–293.1)

* Anti-aaRS = anti–aminoacyl–transfer RNA synthetase; PM = polymyositis; OR = odds ratio; 95% CI = 95% confidence interval (see Table 1 for other definitions).
† Versus patients with anti–MDA-5 antibody–positive DM.

healthy controls, adjusted P value not significant). P values with Bonferroni correction for multiple comparisons less than 0.0023 were considered significant; this was determined by dividing the P value of 0.05 by 22 (the number of HLA genotypes). The inadequate statistical power may be attributed to small sample sizes. Moreover, the frequency of HLA–DRB1*0405 was significantly higher in the patients with anti–MDA-5 antibody–positive DM than in the patients with PM/DM without anti-aaRS antibody or ILD ($P = 0.0003$) or in the healthy controls ($P = 0.00018$). The frequency of HLA–DRB1*0405 was also high in patients with anti-aaRS antibody–positive PM/DM, although it was not significantly different from that in the other subsets. No significant differences were found regarding the frequencies of the other alleles.

Frequency of HLA–DRB1*0101/*0405 in patients with anti–MDA-5 antibody–positive DM compared with other PM/DM patient subsets. In this study, the HLA–DRB1*0101 or *0405 allele was identified in all but 1 of the 17 anti–MDA-5 antibody–positive patients. In the HLA–DRB1 alleles, residues 70–74 of the DRβ chain form the third hypervariable region, an important region for antigen presentation. This amino acid sequence is QRRAA, which is a shared epitope motif in both DRB1*0101 and DRB1*0405. We speculated that QRRAA may be a critical sequence in the pathophysiology of anti–MDA-5 antibody–positive DM. We considered the role of both DRB1*0101 and DRB1*0405 in anti–MDA-5 antibody–positive DM. Therefore, the combined frequency of the DRB1*0101 allele and the DRB1*0405 allele was compared among patients with anti–MDA-5 antibody–positive DM, patients with anti-aaRS antibody–positive PM/DM, and patients with PM/DM without anti-aaRS antibody or ILD.

As shown in Table 3, the combined frequency of DRB1*0101 and *0405 was significantly higher in patients with anti–MDA-5 antibody–positive DM than in patients with PM/DM without anti-aaRS antibody or ILD, with an odds ratio (OR) of 42.7 (95% confidence interval [95% CI] 4.9–370.2, $P = 1.1 \times 10^{-5}$), or in patients with DM without anti-aaRS antibody or ILD (OR 32 [95% CI 3.5–293.1], $P = 2 \times 10^{-4}$). The combined frequency of DRB1*0101 and *0405 was also higher in patients with anti–MDA-5 antibody–positive DM than in patients with anti-aaRS antibody–positive PM/DM (OR 13.3 [95% CI 1.6–112.6], $P = 4.5 \times 10^{-3}$) and patients with anti-aaRS antibody–positive DM (OR 9.3 [95% CI 1.0–86.4], $P = 4.4 \times 10^{-2}$). Moreover, the frequency of these alleles was higher in patients with anti-aaRS antibody–positive PM/DM than in patients with PM/DM without anti-aaRS antibody or ILD (OR 3.2 [95% CI 1.1–8.9], $P = 2.4 \times 10^{-2}$).

DISCUSSION

We have demonstrated an association between a genetic factor and anti–MDA-5 antibody–positive DM. Specifically, this study shows that HLA–DRB1*0101/*0405 is associated with susceptibility to anti–MDA-5 antibody–positive DM. HLA–DRB1*0301 is associated with susceptibility to anti-aaRS antibody–positive PM/DM in Caucasians. In contrast, the frequency of HLA–DRB1*0301 is low, but the frequency of HLA–DRB1*0405 is relatively high, at ~20%, in the Japanese population. HLA–DRB1*0405 is associated with susceptibility to anti-aaRS antibody–positive PM/DM in the Japanese population, whereas HLA–DRB1*0101 is not (8). In the present study, the frequency of HLA–DRB1*0405 was high in both anti–MDA-5 antibody–positive DM and anti-aaRS antibody–positive PM/DM. In contrast, the frequency of HLA–DRB1*0405 among patients with PM/DM without anti-aaRS antibody or ILD was similar to that in healthy controls. Type 1