Table 1 Associations between systemic lupus erythematosus (SLE)-related features and anti-70-kDa polypeptide of the U1-ribonucleoprotein (RNP) complex (U1-70k) antibodies (Abs)

	Anti-U1-70k Abs		P^a	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	Positive ($n = 47$)	Negative ($n = 59$)					
Malar rash/discoid rash	13 (28 %)	19 (32 %)	0.674	41	54	28	68
Oral or nasal ulcers	3 (6 %)	4 (7 %)	1.000	43	56	6	93
Serositis	5 (6 %)	3 (6 %)	0.533	55	57	13	92
Arthritis	13 (28 %)	13 (22 %)	0.650	50	58	28	78
Active nephritis	14 (30 %)	17 (29 %)	1.000	45	56	30	71
CNS lupus	15 (32 %)	17 (29 %)	0.832	47	57	32	71
Thrombocytopenia	5 (11 %)	3 (5 %)	0.462	63	57	11	95
Leukopenia	10 (21 %)	19 (4 %)	0.808	48	56	21	81
Positive anti-dsDNA Ab	22 (47 %)	28 (47 %)	1.000	44	55	47	53
Antiphospholipid Abs[b]	8 (17 %)	11 (19 %)	1.000	42	55	17	81
Positive ANA	47 (100 %)	58 (98 %)	1.000	45	100	100	2

CNS central nervous system, *dsDNA* double-stranded DNA, *ANA* antinuclear antibody, *PPV* positive predictive value, *NPV* negative predictive value

[a] *P* values were determined by Fisher's exact test

[b] Antiphospholipid antibodies include lupus anticoagulant, anti-cardiolipin antibodies, and anti-β2GPI antibodies

Table 2 Associations between systemic lupus erythematosus (SLE)-related features and titers of anti-70-kDa polypeptide of the U1-ribonucleoprotein (RNP) complex (U1-70k) antibodies (Abs)

Feature	Median anti-U1-70k Ab (OD)		P^a
	Present	Absent	
Malar rash/discoid rash	0.06	0.07	0.912
Oral or nasal ulcers	0.03	0.07	0.546
Serositis	0.11	0.06	0.265
Arthritis	0.14	0.06	0.269
Active nephritis	0.07	0.07	0.830
CNS lupus	0.07	0.06	0.568
Thrombocytopenia	0.17	0.06	0.258
Leukopenia	0.07	0.06	0.563
Positive anti-dsDNA Ab	0.07	0.06	0.159
Antiphospholipid Abs[b]	0.07	0.07	0.339
Positive ANA	0.07	0.04	–

CNS central nervous system, *dsDNA* double-stranded DNA, *ANA* antinuclear antibody, *OD* optical density

[a] *P* values were determined by the Mann–Whitney *U* test

[b] Antiphospholipid Abs include lupus anticoagulant, anti-cardiolipin antibodies, and anti-β2GPI antibodies

probably due to the limited sample size. We speculate that patients with high titers of anti-U1-RNP antibodies measured with ELISA but without anti-U1-70k antibodies possess antibodies against U1-A, U1-C, or U1-RNA, although we did not directly measure them. Associations between antibodies against individual U1-RNP proteins and symptoms and/or organ involvement, such as decreased lung diffusion and the presence of antibodies against U1-A and U1-C, have been reported [35]. Sato et al. [23] also reported that the anti-U1-70k index was higher than the anti-U1-A and anti-U1-C indices in the CSF of anti-U1-RNP-antibody-positive SLE patients with CNS syndromes.

Relationships with anti-U1-70k antibodies were different between psychiatric syndromes alone and CNS syndromes as a whole in SLE. Because it is unlikely that a single pathogenic mechanism is responsible for all of these syndromes, different antibodies could be responsible for different NP syndromes in SLE. In fact, diffuse NP manifestations that are practically equivalent to the psychiatric syndromes described in this study have been reported to be linked with other antibodies, such as anti-*N*-methyl-D-aspartate receptor subunit NR2 antibodies and anti-phosphorylated ribosomal (P ribosomal) antibodies [4, 9].

There are a few mechanisms that may explain how serum autoantibodies can be pathogenic in CNS syndromes in SLE patients. Autoantibodies can enter the CSF of SLE patients by passive transfer from the circulatory system through a permeabilized blood–brain barrier [1]. The blood–brain barrier may be permeabilized by factors attributable to SLE (e.g., immune complex deposition and cytokines) or independent of SLE (e.g., smoking and hypertension) [6]. For example, potential interactions between serum anti-P ribosomal antibodies and CSF antineuronal antibodies in the pathogenesis of NP-SLE have been suggested [1, 36]. Antibodies to U1-RNP interact with both mononuclear and endothelial cells [37–39]. These interactions can provide multiple putative pathways for mediation of tissue injury [20]. In this case, therefore, anti-U1-70k antibodies themselves may not be

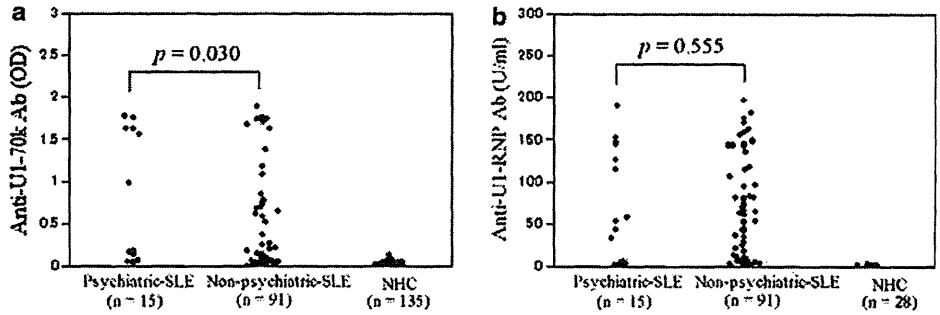

Fig. 4 Comparison of enzyme-linked immunosorbent assay (ELISA) autoantibody titers against 70-kDa polypeptide of the U1-ribonucleoprotein (RNP) complex (U1-70k) (**a**) and U1-RNP (**b**) between systemic lupus erythematosus (SLE) patients with or without psychiatric syndromes (n = 15 and 91, respectively) and between 135 normal healthy controls (NHCs) with anti-U1-70k antibody ELISAs or 28 normal healthy controls with anti-U1-RNP antibody ELISAs. Levels of anti-U1-70k antibodies were significantly elevated in SLE patients with psychiatric disorders compared with SLE patients without psychiatric disorders (p = 0.030 by the Steel–Dwass multiple comparison test) (**a**). By contrast, no significant difference was observed in the levels of serum anti-U1-RNP antibodies in SLE patients with or without psychiatric syndromes (**b**) (p = 0.555)

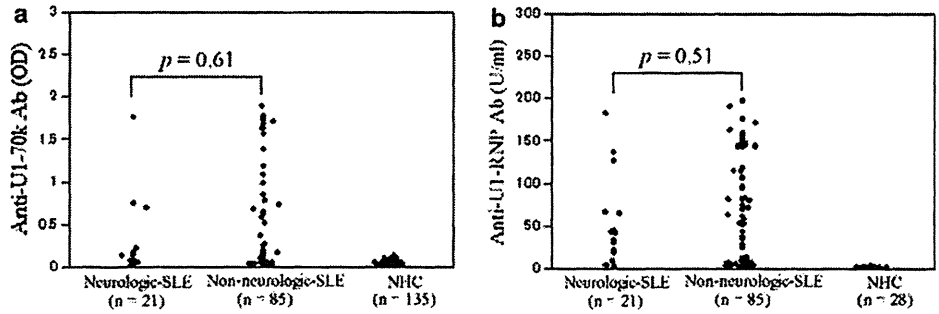

Fig. 5 Comparisons of enzyme-linked immunosorbent assay (ELISA) autoantibody titers against 70-kDa polypeptide of the U1-ribonucleoprotein (RNP) complex (U1-70k) (**a**) and U1-RNP (**b**) between systemic lupus erythematosus (SLE) patients with or without neurologic syndromes (n = 21 and 85, respectively) and between 135 normal healthy controls (NHCs) with anti-U1-70k antibody ELISAs and 28 NHCs with anti-U1-RNP antibody ELISAs. No significant difference was observed in the levels of serum anti-U1-70k antibodies (**a**) or anti-U1-RNP antibodies (**b**) in SLE patients with or without neurologic syndromes (p = 0.61 and 0.51, respectively by the Steel–Dwass multiple comparison test)

pathogenic in the CNS, but they may cause damage to the blood–brain barrier. If other factors increase the permeability of the blood–brain barrier, serum anti-U1-70k antibodies could be directly pathogenic in the CNS. Because the CSF was not sampled in this study, and because no circulating biomarkers of blood–brain barrier permeability were assessed, the question of whether autoantibodies were present within the intrathecal space was not addressed. Moreover, interferogenic activity, the ability of the serum or the CSF to induce interferon (IFN)-α synthesis in the presence of an IFN-producing cell, in the CSF of SLE patients is significantly correlated with serum anti-U1-RNP antibody levels but not with other known antinuclear antibodies [40]. Therefore, anti-U1-RNP antibodies and their immune complexes in the CSF may have pathogenic roles in NP-SLE [23].

In NP-SLE, levels of serum autoantibodies do not always reflect their behavior in the CSF [5]. Actually, Sato et al. [23] reported that the frequency of anti-U1-RNP antibodies in the CSF and the anti-U1-RNP index is higher in SLE patients with CNS syndromes than in those without, whereas no association was observed between the presence of serum anti-U1-RNP antibodies and CNS syndromes in SLE. However, the reason for these conflicting results may be explained simply by our finding of associations between serum anti-U1-70k antibodies and psychiatric syndromes, but not with CNS syndromes as a whole, in SLE patients. Alternatively, this finding may be related to the heterogeneity of patients with CNS lupus or the methodological differences between Sato et al.'s RNA immunoprecipitation assay and our ELISA.

Anti-U1-70k antibodies were not present in serum samples obtained from some SLE patients with psychiatric syndromes, probably because several different autoimmune and inflammatory mechanisms are likely to play roles in the pathogenesis of NP-SLE [6]. Again, a single pathogenic mechanism is unlikely to be responsible for all of them. Lupus-specific mechanisms underlying NP disease include vasculopathy of the intracranial vessels, local or systemic

production of inflammatory mediators, and generation of specific autoantibodies.

Because some elements in this study design may limit its strength, the novel findings, although promising, require cautious interpretation and further investigation before leading to firm conclusions. First, study validity could be improved by a larger sample size and a nonretrospective study design, although our sample size was comparable with that of the majority of previous studies dealing with anti-U1-RNP antibodies or CNS syndromes in SLE patients. Second, because participants were mostly Japanese, it is not clear whether anti-U1-70k antibodies have different effects on psychiatric/CNS syndromes in patients of different ethnic backgrounds. In fact, ethnic differences have been reported in the frequency of end-organ involvement in the Miami MCTD versus the Missouri Caucasian MCTD study groups [16]. Third, although we speculate that patients with high anti-U1-RNP antibody titers but without anti-U1-70k antibodies measured with ELISA also possessed antibodies against U1-A, U1-C, or U1-RNA, some of the serum samples might have reacted with epitopes altered by the U1-RNA binding to U1-70k [33], which we did not assess. Finally, the biological relationship between the ubiquitous protein (i.e., U1-70k) and CNS specificity was not clear, although we speculated above on several possible pathological mechanisms, citing literature information.

In conclusion, despite its limitations, this study suggests that anti-U1-70k antibodies in serum are associated with psychiatric syndromes but not with CNS syndromes as a whole or with neurologic syndromes in SLE patients. The anti-U1-70k antibodies might be involved in pathological mechanisms of SLE psychiatric syndromes. Determining the precise role of anti-U1-70k antibodies in the pathogenesis of CNS syndromes and their usefulness as biomarkers in SLE patients will require further study, including investigations employing animal models.

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Conflict of interest None.

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Original article

Urinary free light chain is a potential biomarker for ISN/RPS class III/IV lupus nephritis

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Abstract

Objectives. To evaluate the use of urinary free light chains (FLCs) as a biomarker for proliferative LN and the potential association between the intensity of plasma cell infiltration of the kidney and urinary FLC levels in LN.

Methods. Forty-three SLE patients were consecutively enrolled in the study. These patients were divided into an International Society of Nephrology and Renal Pathology Society (ISN/RPS) class III/IV LN subset ($n = 18$) and an ISN/RPS class I/II/V (class non-III/IV) LN subset ($n = 25$). The expression of κ-LCs, λ-LCs, CD19 and CD138 in kidney specimens was also evaluated with immunohistochemical staining. To measure FLC levels before and after treatment, an additional six patients with class III/IV LN were consecutively enrolled.

Results. Urinary FLCs were significantly higher in the class III/IV LN subset than in the class non-III/IV LN subset. Urinary λ-FLC levels were significantly correlated with the urinary protein–creatinine ratio in the class III/IV LN subset ($r_s = 0.67$, $P < 0.01$). Moreover, the LC-secreting $CD19^-/CD138^+$ cell counts in the kidney specimens were higher in the class III/IV LN subset than in the class non-III/IV LN subset. Total urinary FLC levels were correlated with the numbers of $CD138^+$ cells in the kidney ($r = 0.71$, $P = 0.03$). Following treatment, urinary λ-FLCs could not be detected in any of the patients.

Conclusion. The intensity of plasma cell infiltration of the kidney is associated with urinary FLC levels. Urinary FLCs are potentially useful biomarkers in ISN/RPS class III/IV LN or proliferative LN.

Key words: lupus nephritis, free light chain, plasma cell, disease activity.

BASIC SCIENCE

Introduction

SLE is an autoimmune disease with multiple organ manifestations, including skin lesions, arthritis, serositis, nephritis and neuropsychiatric and haematological disorders. LN is a common complication of SLE; the frequency of LN is approximately 31–65% among SLE patients in the USA and Europe and 45–86% in Japan [1]. The long-term prognosis for LN has improved [2]. However, WHO class IV LN is one of the most common contributors to end-stage renal failure (ESRF). The frequency of ESRF is 40.9% in patients with WHO class IV LN compared with 2.6% in patients with non-class IV LN [3]. In general, combination therapy with corticosteroids and immunosuppressive agents (IAs), such as cyclophosphamide and mycophenolate mofetil, is recommended for class III/IV LN, as defined by the International Society of Nephrology and the Renal Pathology Society (ISN/RPS). The early diagnosis and appropriate management of ISN/RPS class III/IV LN is critical for improving the renal and overall survival of SLE patients.

Conventional clinical parameters of SLE, such as the levels of serum complement, anti-dsDNA antibodies, creatinine and proteinuria, are assessed to evaluate disease activity and predict complications of ISN/RPS class III/IV LN in SLE patients [4]. However, these markers are not always sufficiently sensitive or specific to detect ongoing disease activity and early relapses of LN. Numerous novel biomarkers, such as serum and urinary cytokines,

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chemokines, adhesion molecules and growth factors, have been evaluated for monitoring treatment response and detecting early renal flares in LN [5]. In particular, urinary biomarkers are more promising than serum biomarkers, possibly because the former result directly from kidney inflammation or injury [5].

It was recently reported that large numbers of anti-dsDNA antibody-secreting plasma cells were present in the kidneys of NZB/W mice and that differentiated (long-lived) plasma cell infiltration of the kidney medulla was associated with more severe LN in SLE patients [6]. A normal immunoglobulin molecule is composed of two light chains (LCs) and two heavy chains. During normal immunoglobulin synthesis by B cells/plasma cells, most LCs bind to heavy chains. Unbound LCs are released from B cells/plasma cells as free light chains (FLCs) [7]. Serum FLC levels are strongly correlated with global disease activity in SLE and may have applications as biomarkers [8]. Furthermore, urinary FLC levels may be useful as quantitative markers of *in vivo* polyclonal B cell activity [9]. Moreover, more severe renal inflammation and a higher risk of disease relapse are associated with increases in urinary FLCs in SLE patients [10, 11]. The findings of the above studies indicate that FLC synthesis by B cells/plasma cells is activated both systematically and locally (in the kidneys) in LN.

However, relationships between serum and urinary FLC levels, associations between serum or urinary FLC levels and pathohistological findings such as ISN/RPS class III/IV LN and class non-III/IV LN and correlations between FLC levels and the infiltration of B cells/plasma cells in the kidney have not yet been evaluated by previous studies in LN. In the present study, we measured both serum and urinary FLC levels and investigated the differences between ISN/RPS class III/IV LN and ISN/RPS class non-III/IV LN at the FLC level.

Materials and methods

Patients

A total of 43 patients with SLE were enrolled in the study. These patients were admitted to our hospital between 2004 and 2006 and consecutively underwent renal biopsies after written informed consent was obtained. All the patients were diagnosed with SLE based on the criteria of the ACR [12] and were admitted to our institution because of active SLE-associated symptoms. Of these 43 patients, 41 were Japanese and 2 were non-Japanese Asians.

To evaluate the serum and urinary FLC levels before and after treatment, another six Japanese patients with ISN/RPS class III/IV LN were consecutively enrolled from 2009 to 2010. This study was approved by the ethics committee of our institution (Institute of Rheumatology, Tokyo Women's Medical University), in accordance with the Declaration of Helsinki.

Data collection

Information that included clinical manifestations and laboratory data was obtained from the patients' medical records. Urinary measurements, including proteinuria and haematuria (by dipstick), urine sediment, protein–creatinine ratio, serum albumin, creatinine, complement components (C3 and C4), IgG and anti-dsDNA antibodies, were evaluated upon admission and prior to the renal biopsy. C3 and C4 were measured using the standard method. Anti-dsDNA antibodies were detected with a radioimmunoassay, with <6 IU/ml considered normal. Antibodies against SS-A, U1-snRNP and Sm were measured with double immunodiffusion. Antibodies against cardiolipin and β2-glycoprotein I were measured with ELISA. The estimated glomerular filtration rate (eGFR) was calculated according to a previously described method using parameters that included serum creatinine levels, age and sex [13]. The SLE disease activity of each patient at admission was assessed using the SLEDAI-2K [14]. The SLEDAI-2K renal scores included urinary casts [haeme-granular or red blood cell (RBC) casts], haematuria [>5 RBC/high-power field (HPF)], proteinuria (urinary protein–creatinine ratio >0.5) and pyuria [>5 white blood cells (WBC)/HPF].

Measurement of serum and urinary FLCs

Sera and spot urine samples were obtained shortly before the renal biopsies were performed and were stored at −80°C. Both serum and urinary FLCs were measured with a nephrometric assay (the Freelite). The normal values for serum κ-FLCs and λ-FLCs were 3.3–19.4 and 5.7–26.3 mg/l, respectively [15]. The normal values for urinary κ-FLCs and λ-FLCs had not been previously determined, although FLCs are present in the urine of healthy individuals only at extremely low concentrations [7]. The FLC values from the spot urine samples correlated well with those from the 24-h urine samples ($r=0.71$, $P<0.01$, data not shown). Therefore the FLC values from the spot urine samples were not corrected for urinary creatinine, as with the urinary protein–creatinine ratio.

Evaluation of renal pathohistology

The renal pathohistological findings were categorized according to the 2003 ISN/RPS classification system [16, 17]. In addition, we evaluated the infiltration of immunoglobulin LC-secreting B cells/plasma cells into the kidney cortex and medulla in ISN/RPS class III/IV LN and compared the measurements with those from the ISN/RPS class V samples. We selected biopsy samples that included sufficient portions of the medulla (with a cortex/medulla proportion <1). Ultimately five ISN/RPS class III/IV samples and four ISN/RPS class V samples fulfilled the criteria described above.

Immunohistochemical staining was performed for κ-LCs (DAKO, Tokyo, Japan, A0191), λ-LCs (DAKO, A0193), CD19 (DAKO, M7296) and CD138 (DAKO, M7228) using the standard method. To evaluate the intensity of the cellular infiltration, positively stained cells were counted in all the fields of the samples. The total cell count of each sample was divided by the entire area of the sample (cell count per mm^2).

Statistical analyses

Statistical analyses were performed using a χ^2 test to compare frequencies, a *t*-test to compare mean values and the Mann–Whitney *U* test to compare median values. Correlation coefficients were calculated as a Pearson's correlation coefficient or Spearman's rank correlation if applicable. The urinary protein–creatinine ratio, the anti-dsDNA antibody levels and the serum and urinary FLC levels before and after treatment were compared using the Wilcoxon signed-rank test. The data were analysed with JMP software (SAS Institute, Cary, NC, USA). *P*-values < 0.05 indicated statistical significance.

Results

Comparison of clinical manifestations between ISN/RPS class III/IV and class non-III/IV LN subsets

The 43 enrolled patients were divided into the following two subsets: an ISN/RPS class III/IV LN subset and an ISN/RPS class I, II or V (class non-III/IV) LN subset. The combined classes III and V and classes IV and V were referred to as class III and class IV, respectively. The frequencies of LN classified as ISN/RPS classes I, II, III, IV and V were 9 (21%), 7 (16%), 8 (19%), 10 (23%) and 9 (21%), respectively. As shown in Table 1, the median age, sex, frequency of prednisolone (PSL) or IA administration and PSL dosage did not differ between the two subsets. The urinary protein–creatinine ratio was higher ($P = 0.01$) and the serum albumin level was lower ($P = 0.03$) in the ISN/RPS class III/IV LN subset than in the ISN/RPS class non-III/IV LN subset. The complement level was lower, and the anti-dsDNA antibody titre and the SLEDAI-2K total score were higher in the ISN/RPS class III/IV LN subset.

Comparison of serum and urinary FLC levels between ISN/RPS class III/IV and class non-III/IV LN subsets

As shown in Table 2, the serum κ-FLC levels were significantly higher in the ISN/RPS class III/IV LN subset than in the ISN/RPS class non-III/IV LN subset, although the median values and interquartile ranges were within the normal limits in each subset. There were no significant differences in serum λ-FLCs between the two subsets.

In contrast, both urinary κ-FLCs and λ-FLCs were significantly higher in the ISN/RPS class III/IV LN subset than in the ISN/RPS class non-III/IV LN subset ($P = 0.02$ for both). In the ISN/RPS class non-III/IV LN subset, no urinary λ-FLCs were detected in 13 (52%) of 25 patients.

Relationship between serum and urinary FLCs

No significant correlations were found between the serum and urinary κ-FLCs in both the ISN/RPS class non-III/IV LN subset and the ISN/RPS class III/IV LN subset (Fig. 1A). In addition, there were no significant correlations between serum and urinary λ-FLCs in either subset (Fig. 1B).

Associations between SLEDAI-2K scores and serum/urinary FLCs

We analysed the correlations between the SLEDAI-2K total or renal score and serum or urinary FLCs in all the

Table 1 Comparison of the clinical manifestations of the ISN/RPS class III/IV LN subset and the class non-III/IV LN subset

	Class III/IV (*n* = 18)	Class non-III/IV (*n* = 25)	*P*-value
Age at renal biopsy, years	32 (23–48)	31 (25–42)	0.66
Female, *n* (%)	18 (100)	24 (96)	1.00
Patients who received PSL, *n* (%)	5 (28)	11 (44)	0.35
Dosage of PSL, mg/day	0 (0–15)	5 (0–17.5)	0.43
Patients who received IA, *n* (%)	1 (6)	2 (8)	1.00
Urinary protein–creatinine ratio	0.85 (0.10–1.54)	0.07 (0.03–0.24)	0.01
Serum albumin, g/dl	3.3 (2.9–3.5)	3.8 (3.3–4.1)	0.03
Serum creatinine, mg/dl	0.54 (0.45–0.59)	0.51 (0.46–0.58)	0.68
eGFR, ml/min/1.73 m^2	115 (89–124)	114 (91–135)	0.79
C3, mg/dl	44.0 (32.5–70.3)	65.0 (44.8–86.5)	0.06
C4, mg/dl	4.0 (2.3–7.5)	9.0 (3.8–15)	0.03
IgG, mg/dl	1956 (1635–2706)	1908 (1467–2564)	0.64
Anti-dsDNA Ab, IU/ml	298.5 (150.3–623.8)	20.5 (7.5–29.3)	<0.01
Anti-SS-A Ab positivity, *n* (%)	11 (61)	10 (40)	0.22
Anti-U1 snRNP Ab positivity, *n* (%)	6 (33)	15 (60)	0.12
Anti-Sm Ab positivity, *n* (%)	1 (6)	10 (40)	0.01
Anti-CLβ2GP I Ab positivity, *n* (%)	2 (11)	2 (8)	1.00
SLEDAI-2K total score	15 (10–21)	9 (4–12)	0.01

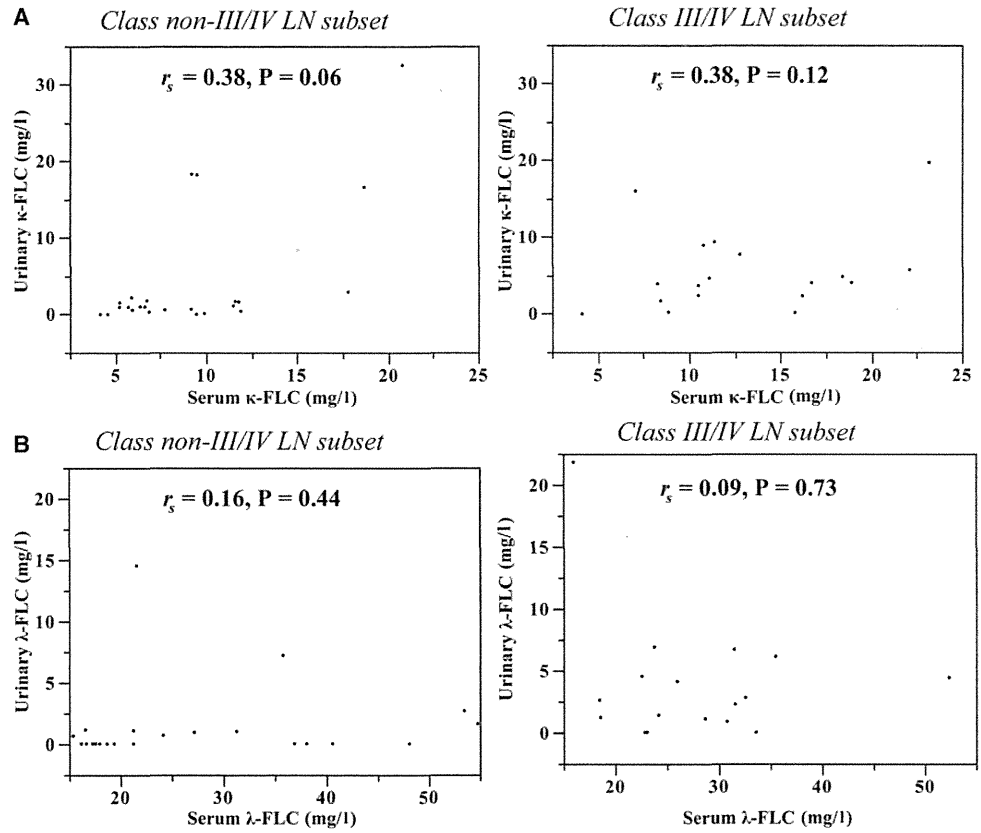
Except for the *n* (%) values, all values listed above represent median (IQR). *P*-values were established using a χ^2 test or Mann–Whitney *U* test. CLβ2GP I: cardiolipin and β2-glycoprotein I; SLEDAI-2K: systemic lupus erythematosus disease activity index 2000; SLEDAI-2K renal score: SLEDAI score with renal involvement, including urinary casts, haematuria, proteinuria and pyuria.

TABLE 2 Comparison of serum and urinary FLC levels in the ISN/RPS class III/IV subset and the ISN/RPS class non-III/IV subset

	Class III/IV ($n=18$)	Class non-III/IV ($n=25$)	*P*-value
Serum κ-FLC (mg/l)	11.3 (8.8–17.1)	9.2 (5.9–11.7)	0.04
Serum λ-FLC (mg/l)	27.4 (22.8–32.9)	21.3 (17.0–36.4)	0.13
Urinary κ-FLC (mg/l)	4.05 (2.16–8.01)	1.0 (0.47–1.99)	0.02
Urinary λ-FLC (mg/l)	2.69 (1.04–6.25)	0 (0–1.11)	0.02

Values listed above represent median (IQR). *P*-values were established using the Mann–Whitney *U* test.

FIG. 1 Associations between serum and urinary FLCs in the ISN/RPS class non-III/IV LN subset and in the ISN/RPS class III/IV LN subset.



An association between serum κ-FLC and urinary κ-FLC (**A**) and an association between serum λ-FLC and urinary λ-FLC (**B**) in each subset.

43 enrolled patients. The serum FLC levels were not significantly correlated with the SLEDAI-2K total score (κ-FLC: $r_s=0.13$, $P=0.40$ and λ-FLC: $r_s=0.20$, $P=0.20$). There was a correlation between the SLEDAI-2K total score and the urinary λ-FLC levels ($r_s=0.40$, $P=0.02$), although no significant correlation existed between the SLEDAI-2K total score and the urinary κ-FLC levels (κ-FLC: $r_s=0.23$, $P=0.15$) and between the SLEDAI-2K renal score and urinary κ- or λ-FLC levels. Moreover, there was no significant difference in serum or urinary FLC levels between the active disease (SLEDAI-2K total score >4) subset and the inactive disease subset in either the ISN/RPS class non-III/IV LN subset and the class III/IV LN subset.

Association between urinary FLCs and conventional biomarkers

Significant correlation was found between urinary λ-FLC and anti-dsDNA Ab ($r_s=0.42$, $P=0.01$), although there was no significant correlation between urinary λ-FLC

and C3 ($r_s = -0.14$, $P = 0.44$) in the ISN/RPS class III/IV subset. There were no statistically significant differences in the urinary FLC levels between the presence of active urinary sediments (RBC > 5/HPF, WBC > 5/HPF or haeme-granular or RBC casts) subset and the absent subset.

Correlation between urinary FLCs and urinary protein–creatinine ratio

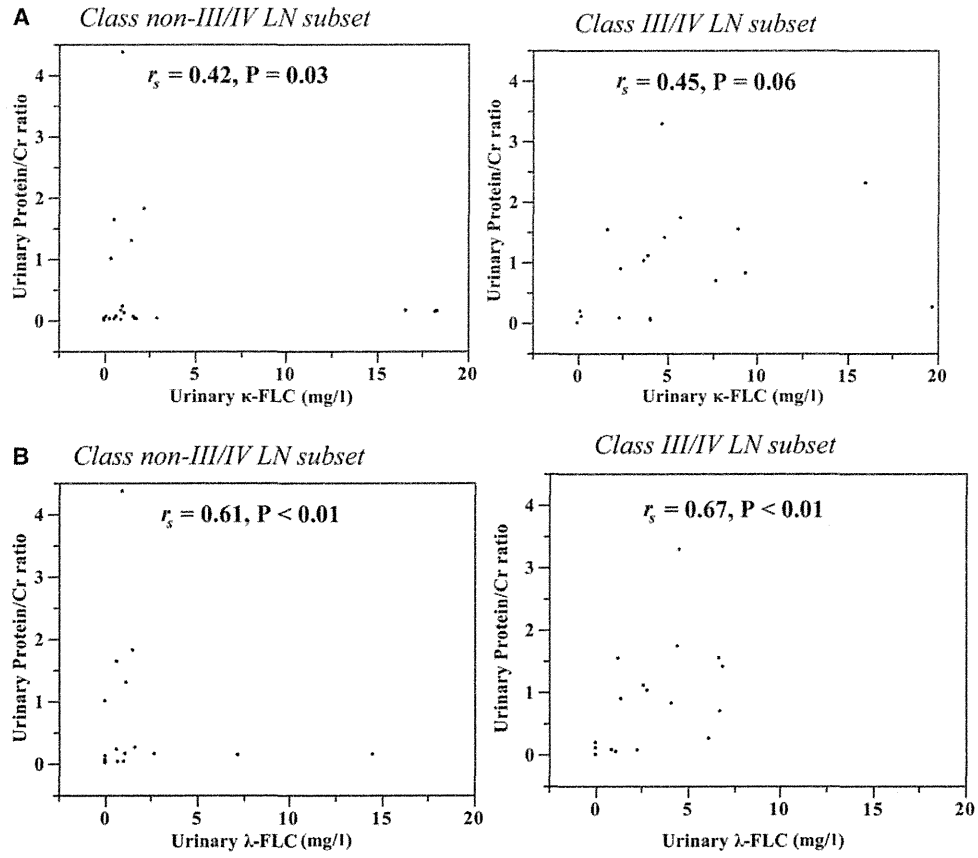
The serum κ-FLCs and λ-FLCs were not correlated with the urinary protein–creatinine ratio in either the ISN/RPS class non-III/IV LN subset or the ISN/RPS class III/IV LN subset. As shown in Fig. 2A, there was also no significant association between the urinary κ-FLCs and the urinary protein–creatinine ratio in the ISN/RPS class III/IV LN subset ($r_s = 0.45$, $P = 0.06$), although the urinary κ-FLCs correlated with the urinary protein–creatinine ratio in the ISN/RPS class non-III/IV LN subset ($r_s = 0.42$, $P = 0.03$). The urinary λ-FLCs were significantly positively correlated with the urinary protein–creatinine ratio in both the ISN/RPS class non-III/IV LN subset ($r_s = 0.61$, $P < 0.01$) and the ISN/RPS class III/IV LN subset ($r_s = 0.67$, $P < 0.01$) (Fig. 2B). On the other hand, the C3 and anti-dsDNA antibody levels were not associated with the urinary protein–creatinine ratio.

To investigate an association between the levels of urinary FLC and proteinuria in detail, we compared the ISN/RPS class III/IV subset with the class V subset. The median value of the urinary protein–creatinine ratio was 0.8 and 0.9 in the ISN/RPS class III/IV subset and the class V subset, respectively. There was no statistically significant difference in the urinary protein–creatinine ratio between the two subsets. In contrast, the ratio of the urinary κ- or λ-FLC levels to the urinary proteinuria concentration was higher in the class III/IV subset than in the class V subset ($P = 0.05$ and 0.01 in urinary κ-FLC and urinary λ-FLC, respectively).

Immunohistochemical staining of kidney specimens with immunoglobulin light chains, CD19 and CD138 in ISN/RPS classes III/IV and V LN

To compare the ISN/RPS class III/IV LN patients ($n = 5$) with the ISN/RPS class V LN patients ($n = 4$) in terms of the infiltration of B cells/plasma cells and the status of the LCs synthesized by those cells in the kidney, immunohistochemical staining was performed for κ-LCs, λ-LCs,

FIG. 2 Correlations between urinary protein–creatinine ratios and urinary FLCs in the ISN/RPS class non-III/IV LN subset and the ISN/RPS class III/IV LN subset.



Correlations between (**A**) the urinary protein–creatinine ratio and urinary κ-FLC and (**B**) the urinary protein–creatinine ratio and urinary λ-FLC in each subset.

CD19 and CD138 in both patient subsets. As shown in a representative ISN/RPS class III/IV LN case (Fig. 3A), κ-LCs and λ-LCs were synthesized mainly by $CD138^+$ cells. The κ-LC^+ cell counts were significantly higher ($P = 0.03$) in the ISN/RPS class III/IV LN subset than in the ISN/RPS class V LN subset (Fig. 3B). In addition, the λ-LC^+ cell counts and the CD 138^+ cell counts were higher in the class III/IV LN subset than in the class V LN subset, although this difference was not significant (Fig. 3C and E). The $CD19^+$ cell counts did not differ between the two subsets (Fig. 3D). Among all of the infiltrating $CD138^+$ cells, there were no significant differences between the κ-LC^+ cell count and the λ-LC^+ cell count. Moreover, the $CD138^+$ cells expressed virtually no CD19 in the kidney specimens from the ISN/RPS class III/IV LN subset. Almost all of the $CD138^+$ cells were located around the glomeruli or the tubulointerstitium of the margin between the renal cortex and the medulla.

Correlation between urinary FLC levels and the number of $CD138^+$ cells infiltrating the kidney

As shown in Fig. 3F, the total (κ + λ) urinary FLC levels were significantly correlated ($r = 0.80$, $P < 0.01$) with the numbers of $CD138^+$ cells infiltrating the kidney of nine patients described above (five patients with ISN/RPS class III/IV LN and four with ISN/RPS class V LN), although there were no such associations with the total serum FLC levels.

Comparison of clinical parameters before and after immunosuppressive treatment

As shown in Fig. 4A and B, the urinary protein–creatinine ratio and the anti-dsDNA antibody titre were lower after treatment than before treatment ($P = 0.03$ and 0.06, respectively) in six patients with ISN/RPS class III/IV LN. Both serum κ-FLCs and serum λ-FLCs decreased, although their values were almost within normal limits prior to treatment (Fig. 4C and D). Both urine κ-FLCs and urine λ-FLCs also decreased after treatment compared with before treatment (Fig. 4E and F). Notably, no urine λ-FLCs could be detected in any patient after treatment.

Discussion

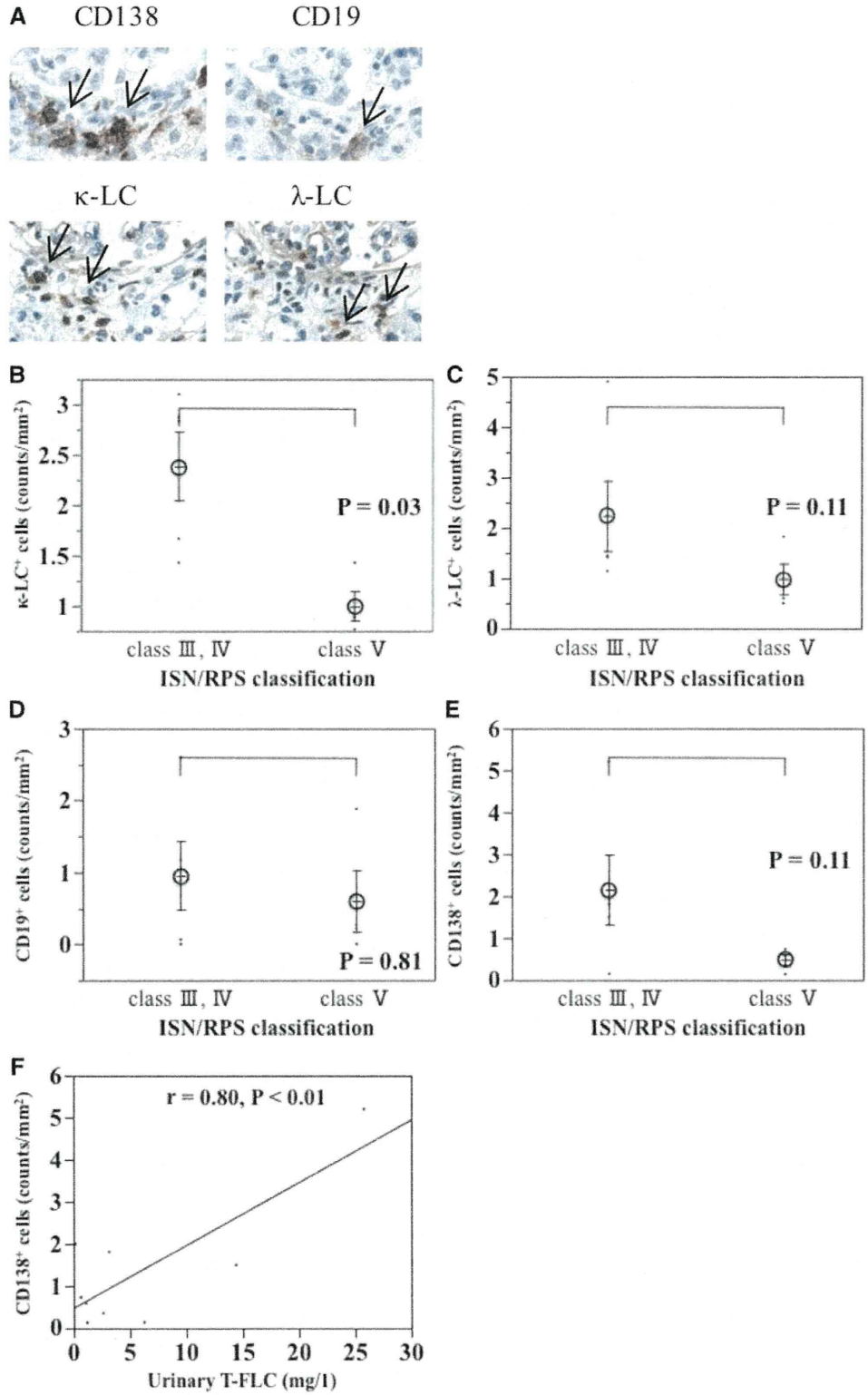
The present study demonstrated that urinary FLC levels were elevated and were associated with the intensity of plasma cell infiltration of the kidney in ISN/RPS class III/IV LN patients. During normal immunoglobulin synthesis, B cells/plasma cells release FLCs, which pass through the glomerular filtration barrier rapidly (with a serum half-life of 2–6 h) [7]. The urinary FLC levels are low in healthy individuals [18, 19]. Serum FLC levels are elevated in many autoimmune/inflammatory diseases, and FLC levels are strongly correlated with other markers of B cell/plasma cell activation [8, 20, 21]. Urinary FLC levels might represent quantitative markers of real-time, *in vivo* polyclonal B cell/plasma cell activity; SLE relapse has been associated with increases in urinary FLCs [9, 10]. According to the previous report described by Tsai *et al.* [11], urinary FLCs were not detected in patients with inactive LN (24-h urinary excretion; range 3–20 g/day). In the present study, the ratio of the urinary FLC levels to the proteinuria levels was higher in the ISN/RPS class III/IV LN subset than the ISN/RPS class V LN subset, although there was no significant difference in the levels of proteinuria between the two subsets. These results indicate that urinary FLC levels led to not only the synthesis of FLCs or the urinary protein excretion concentrations, but also the disease activity of LN. Urinary FLC levels may better reflect the disease activity of LN in real time than conventional markers.

A recent study revealed that plasma cell infiltration into the renal medulla is correlated with the amount of inflammation and the disease severity of patients with LN. A study of NZB/W mice revealed that anti-dsDNA antibodies were produced by long-lived plasma cells located in the kidney [6]. $CD138^+$ cells that express low levels of CD19 are compatible with a long-lived plasma cell phenotype [22]. Long-lived plasma cells are capable of continuously secreting antibodies. These cells live primarily in the bone marrow and secondarily in niches in the spleen and chronically inflamed tissues, such as the kidneys in SLE or the synovia in RA [23]. The present study demonstrated that LCs were synthesized mainly by $CD19^-/CD138^+$ cells and that the total urinary FLC levels were significantly correlated with the numbers of kidney-infiltrating $CD138^+$ cells in ISN/RPS class III/IV LN. In contrast, the ISN/RPS class non-III/IV (e.g., class V) LN patients exhibited low or undetectable urinary FLC levels, and the $CD138^+$ cells infiltrating their kidneys were scarcely detectable. These findings revealed that the infiltration of long-lived plasma cells into the kidney may play a role in local inflammation and that the urinary FLC levels reflect the intensity of the plasma cell infiltration of the kidney in proliferative types of LN, such as ISN/RPS class III/IV LN.

In the present study, urinary λ-FLCs were not detected after immunosuppressive therapy in any of the ISN/RPS class III/IV LN patients. In contrast, urinary κ-FLC levels were significantly decreased (but detectable) after treatment, although there was no significant difference between κ-$LC^+/CD138^+$ cell counts and λ-$LC^+/CD138^+$ cell counts in the ISN/RPS class III/IV LN kidney specimens. κ-LCs and λ-LCs are encoded by different chromosomes. λ-FLCs have a dimeric structure, whereas κ-FLCs have a monomeric structure [24]. In amyloid light chain amyloidosis, λ-LCs are involved in amyloid deposition more often than κ-LCs [25]. Although the precise molecular functions of each type of FLC remain unknown, the molecular differences between κ-LCs and λ-LCs might cause the discrepancies in the serum and urinary levels between κ-FLC and λ-FLC.

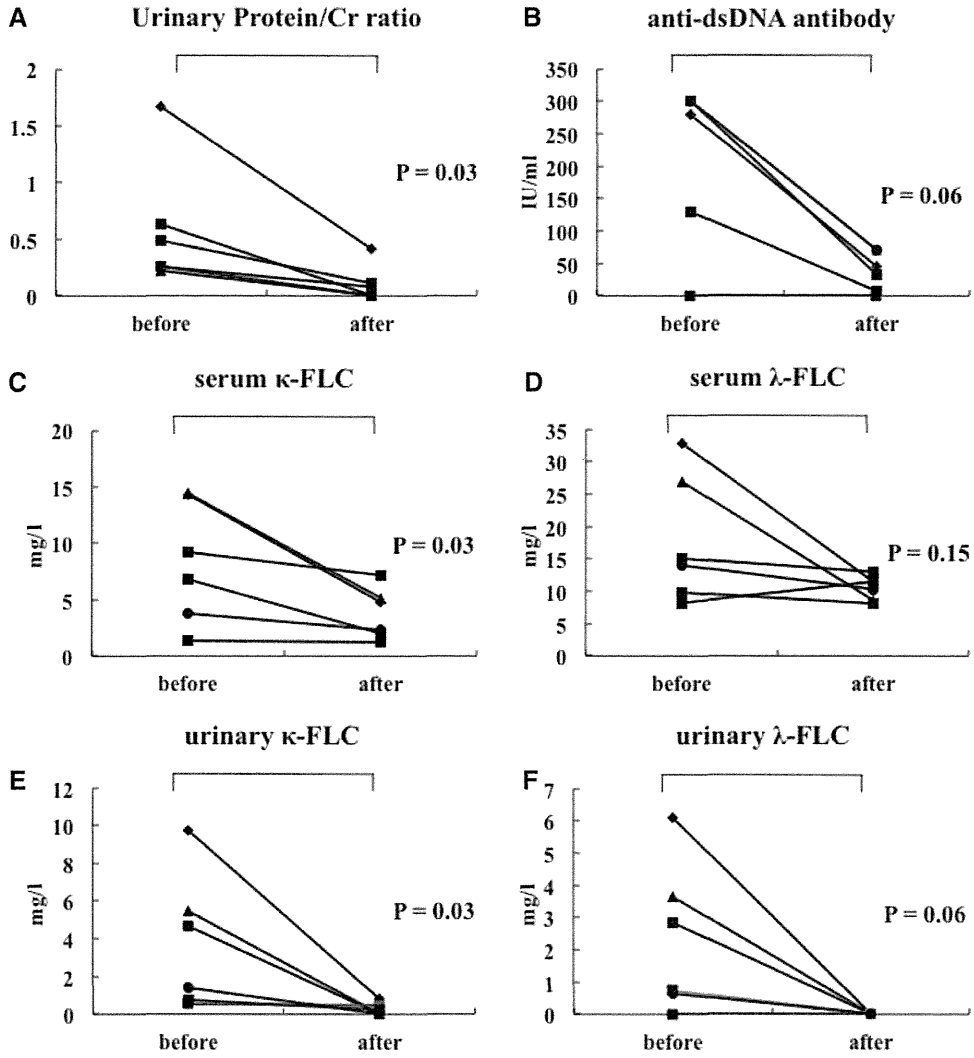
A previous study reported that serum FLC levels were correlated with global disease activity in SLE [8]. However, the present study found no correlation between the SLEDAI-2K scores and the serum FLC levels. A bias in the selection of enrolled patients may have contributed to the differences between the results of the previous study and ours. In this study, urinary FLC levels were correlated with the urinary protein–creatinine ratio, although

Fig. 3 Immunohistochemical staining for immunoglobulin LCs, CD19 and CD138 in ISN/RPS class III/IV and class V LN kidney specimens.



In a representative ISN/RPS class III/IV LN case, κ-LC and λ-LC were synthesized mainly by $CD138^+$ cells (**A**). Immunohistochemical staining for κ-LC (**B**), λ-LC (**C**), CD19 (**D**) and CD138 (**E**) in the class III/IV LN subset ($n = 5$) and in the class V LN subset ($n = 4$). There was a positive correlation between the total urinary FLC levels and the numbers of $CD138^+$ cells infiltrating the kidney (**F**).

FIG. 4 Clinical parameters before and after treatment.



Urinary protein–creatinine ratios (**A**), anti-dsDNA antibody titres (**B**), serum κ-FLC levels (**C**), serum λ-FLC levels (**D**), urinary κ-FLC levels (**E**) and urinary λ-FLC levels (**F**).

there were no significant correlations between the urinary protein–creatinine ratio and serum biomarkers such as FLC, anti-dsDNA Ab and C3. Urinary FLC could reflect the activity of LN. However, urinary FLC is not a specific marker for LN. Urinary FLC may be increasing in the other glomerulonephritis involved with plasma cells infiltration. Moreover, serum and urinary FLC levels can be influenced by several factors, including renal dysfunction and hypergammopathies such as infections and multiple myeloma [7, 10].

There are some limitations to our study. First, the serum and urine samples were stored for long term. This might affect the results of the serum and urinary FLC levels. Second, the sample size was small. We did not longitudinally evaluate the urinary FLC level after treatment. So we are unsure whether urinary FLC is a useful biomarker as a predictor for renal relapse.

In conclusion, urinary FLC is a non-invasive biomarker for ISN/RPS class III/IV LN or proliferative LN. The intensity of the plasma cell infiltration of the kidney is also associated with urinary FLC levels in LN.

Rheumatology key messages

- Urinary FLCs are useful as a non-invasive biomarker for the response to treatment of ISN/RPS class III/IV LN.
- The intensity of the plasma cell infiltration of the kidney is associated with urinary FLC levels in LN.

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Original article

Anti-MDA5 antibody, ferritin and IL-18 are useful for the evaluation of response to treatment in interstitial lung disease with anti-MDA5 antibody-positive dermatomyositis

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Abstract

Objective. The aim of this study was to investigate the precise clinical characteristics and to analyse the association between the anti-MDA5 antibody (anti-MDA5ab) titre and disease status in patients with anti-MDA5ab-positive DM.

Methods. Twenty-seven patients who presented with DM and were positive for the anti-MDA5ab were enrolled. The association between the clinical manifestations and the clinical parameters, including the anti-MDA5ab, was analysed.

Results. The complication of rapidly progressive interstitial lung disease (RP-ILD) occurred in 20 (74%) patients. The frequencies of fatal outcome, relapse and malignancy were 33, 4 and 4%, respectively. Remarkably, a fatal outcome occurred within the first 6 months. Compared with six non-RP-ILD patients, elderly age at onset, severely involved pulmonary function and high levels of serum ferritin were present in 20 RP-ILD patients with anti-MDA5ab. Alveolar–arterial oxygen difference ($AaDO_2$) $\geqslant$32 mmHg and ferritin $\geqslant$828 ng/ml at admission were poor prognostic factors in RP-ILD patients with anti-MDA5ab-positive DM. The median value of the anti-MDA5ab titre on admission was higher in patients who later died than in those who survived. The efficacy of treatment was indicated by the anti-MDA5ab, ferritin and IL-18 concentrations. The decline index of the anti-MDA5ab titre after treatment was lower in the subset of patients who died than in the subset of patients who lived. Sustained high levels of anti-MDA5ab, ferritin and IL-18 were present in the patients who died.

Conclusion. Anti-MDA5ab titre and ferritin and IL-18 concentrations are useful for the evaluation of the response to treatment and the status of ILD in patients with anti-MAD5ab-positive DM.

Key words: dermatomyositis, interstitial lung disease, anti-MDA5 antibody, ferritin, interleukin-18.

BASIC

Introduction

DM is characterized by inflammation of the skin and muscles [1]. Rapidly progressive interstitial lung disease (RP-ILD) in particular is of prime importance in the clinical management of patients with DM because it is an intractable and life-threatening complication [2–5]. Clinically amyopathic DM (CADM) includes typical skin lesions with amyopathy or hypomyopathy [6]. CADM patients with the anti-MDA5 antibody (anti-MDA5ab) frequently develop the complication of RP-ILD, especially in Japan [7–10]. Sato *et al.* [7] identified melanoma differentiation-associated gene 5 (MDA5) as the CADM-140 antigen. The MDA5 protein plays a role in the innate immune response. MDA5 initially recognizes picornaviruses and evokes antiviral responses by eliciting the production of type I IFNs

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and TNF-α [11]. We previously reported that high levels of ferritin are associated with the development and prognosis of RP-ILD with DM [9, 12]. In addition, IL-18 is a potential contributor to ILD with DM [13]. High levels of ferritin and IL-18 are also implicated in macrophage activation syndrome (MAS) [14, 15]. Although a cytokine storm may contribute to the pathogenesis of RP-ILD with anti-MDA5ab-positive DM, especially in the skin and lungs, the precise pathogenesis remains unknown. Moreover, long-term prognosis, frequency of recurrence, complication with malignancy and the association between the anti-MDA5ab titre and the clinical course remain unclear in anti-MDA5ab-positive DM.

Thus we investigated the clinical characteristics and the correlation between the anti-MDA5ab titre and clinical parameters, such as ferritin and IL-18 levels, in patients with anti-MDA5ab-positive DM. In addition, we analysed the association between the anti-MDA5ab titre and the clinical course in these patients.

Patients and methods

Patients

The present retrospective study included patients with idiopathic inflammatory myopathy who were admitted to the Tokyo Women's Medical University Aoyama Hospital or Keio University Hospital from August 1992 to December 2009. All of the enrolled patients suffered from skin rash, myopathy or respiratory symptoms (or a combination thereof) at admission. These patients were diagnosed with DM or CADM based on the criteria of Bohan and Peter [16] or Sontheimer [17], respectively. In general, CADM presents with typical skin lesions and either amyopathy or hypomyopathy for >6 months. A subset of the CADM group included patients who developed fatal ILD within the first 6 months of this study. Medical records were obtained from 142 and 53 patients who were diagnosed with DM and CADM, respectively. In the present study, 5 DM patients and 22 CADM patients who were positive for the anti-MDA5ab were enrolled. The frequencies of anti-MDA5ab positivity were 4 and 42% in the DM patients and in the CADM patients, respectively. Clinical data were obtained from medical records on admission. The study was approved by the ethical committee of the Institute of Rheumatology, Tokyo Women's Medical University, and the study complied with the Declaration of Helsinki guidelines. Disease duration was defined as the time between the appearance of symptoms, such as skin rash, myopathy or respiratory symptoms, and the initiation of treatment.

Evaluation of clinical laboratory parameters and the anti-MDA5ab

Blood tests evaluated creatine kinase (CK), lactate dehydrogenase (LD), KL-6, CRP, ferritin and ANA. Serum IL-18 was measured with an ELISA (R&D Systems, Minneapolis, MN, USA). The median level (range) of IL-18 was 50.5 (18–121) pg/ml in 30 healthy controls. Anti-MDA5ab was detected with an ELISA using recombinant MDA-5 as an antigen, as described previously [7]. The normal value for the anti-MDA5ab titre was ⩽8 U/ml.

Evaluation of pulmonary function and classification of ILD

The PaO_2/F_iO_2 (P/F ratio), pulse oximeter oxygen saturation/FiO_2 (S/F ratio), alveolar–arterial oxygen difference ($AaDO_2$), forced expiratory volume in 1 s (FEV1)/forced vital capacity (FVC) ratio, VC percentage (%VC) and diffusing capacity of the lung for carbon monoxide (DLco) were used to evaluate pulmonary function. The normal values are defined as >380 for the P/F ratio, >450 for the S/F ratio, <10 mmHg for $AaDO_2$, >70% for the FEV1/FVC ratio, >80% for %VC and >20 ml/min/mmHg for the DLco. The ILD was assessed with chest radiography and CT or high-resolution CT of the chest. RP-ILD is defined as a progressive ILD within 3 months of the onset of respiratory symptoms. Chronic ILD is defined as an asymptomatic, non-rapidly progressive ILD or slowly progressive ILD over 3 months by the International Consensus Statement of Idiopathic Pulmonary Fibrosis of the American Thoracic Society and the European Respiratory Society [18].

Statistical analysis

Statistical analyses were performed using the Student's *t*-test to compare mean values, the Mann–Whitney U-test to compare median values and Fisher's exact test to compare frequencies. Correlation coefficients were established by employing Spearman's correlation coefficients. The cumulative survival rate was calculated using the Kaplan–Meier test. The Wilcoxon signed-rank test was performed when comparing clinical parameters upon admission with those parameters after treatment in each patient. The data were analysed using JMP® software (SAS Institute, Cary, NC, USA). A value of $P < 0.05$ indicated statistical significance.

Results

Clinical characteristics in patients with anti-MDA5ab-positive DM

The clinical characteristics of 27 patients with anti-MDA5ab-positive DM are shown in Table 1. The laboratory data were obtained at the first examination upon admission. The frequency of CADM was 81%. The median value of CK was 92 IU/l (interquartile range: 67–271). The complication of RP-ILD was present in 20 (74%) patients. Six additional patients had the complication of chronic ILD, and one patient had neither complication. Although the values of FEV1/FVC ratio and %VC were normal in almost all patients, the DLco levels were decreased. The median values of KL-6, CRP, ferritin and IL-18 were high. ANA positivity was found in four patients (homogeneous and speckled pattern in two patients, homogeneous pattern in one patient and nucleolar pattern in one patient). In 9 of the other 23 patients without ANA positivity, a cytoplasmic pattern was revealed. The frequencies of the fatal

TABLE 1 Clinical characteristics of patients with anti-MDA5ab-positive DM ($n=27$)

Characteristic	Value
Age, years	48 (13)
Female, *n* (%)	20 (74)
Disease duration, weeks	6 (3-8)
CADM, *n* (%)	22 (81)
RP-ILD, *n* (%)	20 (74)
P/F ratio	348 (324-438)
$AaDO_2$, mmHg	26.2 (10.2-41.5)
FEV1/FVC ratio ($n=18$)	82 (78-89)
%VC ($n=22$)	76 (71-84)
DLco, ml/min/mmHg ($n=9$)	10 (9.1-13.6)
LD, IU/l	382 (253-512)
KL-6, U/ml (normal value $\leqslant$ 500) ($n=23$)	735 (570-985)
CRP, mg/dl	0.63 (0.13-1.37)
Ferritin, ng/ml	642 (217-1120)
IL-18, pg/ml (normal range 18-121) ($n=21$)	550 (328-746)
ANA $\geqslant$160×, *n* (%)	4 (15)
Fatal outcome, *n* (%)	9 (33)
Relapse, *n* (%)	1 (4)
Malignancy, *n* (%)	1 (4)

The values of age indicate the mean (S.D.), and the laboratory markers and pulmonary function tests are presented as the median (interquartile range).

outcome, relapse and malignancy were 33, 4 and 4%, respectively.

Comparison of clinical manifestations between patients with anti-MDA5ab-positive DM with and without RP-ILD

Clinical manifestations were compared between patients who had anti-MDA5ab-positive DM with and without RP-ILD (Table 2). The following information indicates the significant results for the patients with RP-ILD: elderly age at onset ($P=0.0021$), decreased P/F ratio ($P=0.0079$), increased $AaDO_2$ ($P=0.0031$), increased ferritin ($P=0.036$) and high frequency of fatal outcome ($P=0.036$). The median values of %VC and DLco were lower in patients with RP-ILD than in those without RP-ILD, although the difference was not statistically significant. The ferritin level was significantly higher in the patients with RP-ILD. The frequency of fatal outcome was high: 45% in the patients with RP-ILD. The cut-off value as a predictor for RP-ILD was estimated by a receiver operating characteristic (ROC) curve of age at onset, P/F ratio, $AaDO_2$ and ferritin. The following parameters can be used as cut-off values (odds ratio, *P*-value): age $\geqslant$46 years (14, 0.011), P/F ratio <438 torr (23, 0.0047), Aa DO_2 $\geqslant$22 mmHg (34, 0.0017) and ferritin $\geqslant$217 ng/ml (48, 0.0014).

No association between anti-MDA5ab titre and clinical parameters

Correlation coefficients between the anti-MDA5ab titre and clinical parameters were established in patients with anti-MDA5ab-positive DM. The clinical parameters included $AaDO_2$, %VC and laboratory markers (KL-6, CRP, ferritin and IL-18). All of these clinical parameters were obtained from 18 patients at the first examination upon admission. There was no significant correlation between anti-MDA5ab titre and other clinical parameters. Significant correlations were only found between $AaDO_2$ and ferritin ($r_s=0.47$, $P=0.014$) in patients with anti-MDA5ab-positive DM.

Comparison of clinical manifestations in living patients and patients who died with RP-ILD with anti-MDA5ab-positive DM

We analysed the clinical manifestations of the patients who had anti-MDA5ab-positive DM and died, and compared them with the manifestations of the surviving anti-MDA5ab-positive DM patients with RP-ILD (Table 3). The P/F ratio and $AaDO_2$ on admission were significantly worse and the ferritin levels were significantly higher ($P=0.017$) in the patients who died. The median anti-MDA5ab titre was higher, although not significantly ($P=0.099$), in patients who died than in those who survived. RP-ILD was refractory and progressive in the patients who died, although almost all of these patients received combination therapy, including prednisolone (PSL), i.v. CYC therapy (IVCY) and calcineurin inhibitor (CNI). The cut-off values as a predictor of fatal outcome in RP-ILD were estimated by the ROC curve of the P/F ratio, $AaDO_2$ and ferritin and are as follows (odds ratio, *P*-value): P/F ratio < 324 torr (9.3, 0.035), Aa DO_2 $\geqslant$32 mmHg (9.3, 0.035) and ferritin $\geqslant$828 ng/ml (14, 0.025).

Survival rates of patients with anti-MDA5ab-positive DM

The cumulative 100-month survival rate was 66% for the entire anti-MDA5ab-positive DM patient group (Fig. 1A). Fatal outcome occurred remarkably often within the first 6 months. The median survival duration was 2 months in the nine patients who died. In contrast, the median survival duration was 29 months in the 18 surviving patients. Next, the patients with anti-MDA5ab-positive DM were divided into an RP-ILD subset and a non-RP-ILD subset. As shown in Fig. 1B, the cumulative 100-month survival rates were significantly lower in the RP-ILD subset than in the non-RP-ILD subset (log-rank test, $P=0.039$).

Association between the anti-MDA5ab titre and the clinical course in patients with anti-MDA5ab-positive DM

We investigated the association between the clinical parameters and the clinical course. Clinical parameters included the anti-MDA5ab titre, the S/F ratio, KL-6, ferritin and IL-18 concentrations. Seventeen patients with anti-MDA5ab-positive DM, including 15 patients with RP-ILD and 2 patients with chronic ILD, were enrolled. Eleven patients were categorized as the living subset and the remaining six patients formed the dead subset. All six patients in the dead subset had the complication of

Table 2 Comparison of the clinical manifestations between patients with anti-MDA5ab-positive DM with and without RP-ILD

Variable	RP-ILD (–) ($n=7$)	RP-ILD (+) ($n=20$)	*P*
Age, years	35 (4)	52 (2)	0.0021
Female, *n* (%)	6 (86)	14 (70)	0.63
Disease duration, weeks	8 (6–16)	4 (2–8)	0.098
CADM, *n* (%)	6 (86)	16 (80)	1
CK, IU/l	165 (84–271)	85 (47–345)	0.36
LD, IU/l	472 (221–643)	373 (267–500)	0.51
P/F ratio	448 (348–522)	339 (308–388)	0.0079
$AaDO_2$, mmHg	4 (0–18)	30 (24–54)	0.0031
%VC	82 (74–98) ($n=6$)	76 (67–82) ($n=16$)	0.21
DLco, ml/min/mmHg	14.7 (12.5–16.9) ($n=2$)	9.6 (8.9–12.7) ($n=7$)	–
KL-6, U/ml (normal value $\leqslant$500)	346 (278–1104) ($n=5$)	801 (675–1009) ($n=18$)	0.1
CRP, mg/dl	0.46 (0.02–0.80)	0.72 (0.15–1.85)	0.22
Ferritin, ng/ml	186 (120–626)	835 (285–1480)	0.036
IL-18, pg/ml (normal range 18–121)	550 (216–736) ($n=5$)	552 (243–765) ($n=16$)	0.65
Anti-MDA5ab, U/ml	258.8 (217.1–542.7)	152.3 (56.7–376.8)	0.21
Fatal outcome, *n* (%)	0 (0)	9 (45)	0.036

The values of age indicate the mean (S.D.), and laboratory markers and pulmonary function tests are presented as the median (interquartile range).

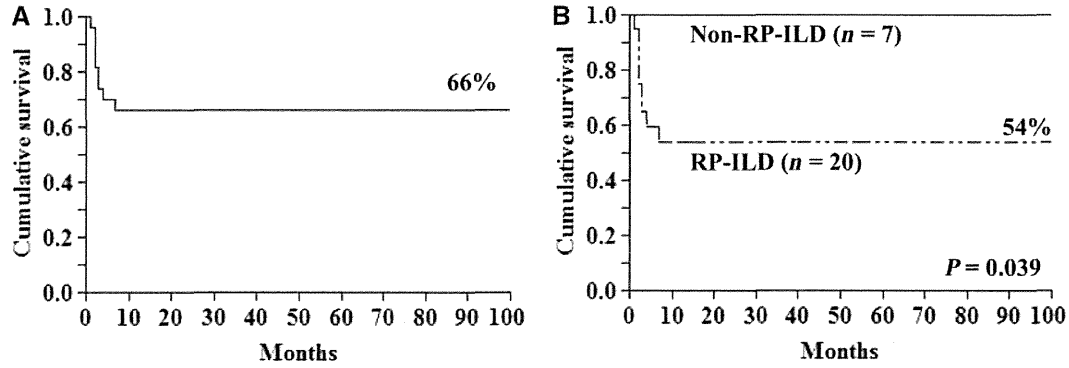
Table 3 Comparison of the clinical manifestations between living patients (alive) and patients who died (dead) with RP-ILD and anti-MDA5ab-positive DM upon admission

Variable	Alive ($n=11$)	Dead ($n=9$)	*P*
Age, years	50 (3)	54 (3)	0.29
Female, *n* (%)	9 (82)	5 (56)	0.34
Disease duration, weeks	8 (2–12)	4 (3–7)	0.76
CADM, *n* (%)	9 (82)	7 (78)	1
CK, IU/l	95 (38–383)	77 (62–324)	0.91
P/F ratio	369 (331–403)	319 (246–352)	0.03
$AaDO_2$, mmHg	26 (22–34)	41 (30–102)	0.044
%VC	76 (71–90) ($n=10$)	71 (62–78) ($n=6$)	0.25
DLco, ml/min/mmHg	9.5 (7.8–13.2) ($n=4$)	10 (8.9–12.7) ($n=3$)	–
LD, IU/l	364 (243–488)	460 (308–518)	0.3
KL-6, U/ml (normal value $\leqslant$500)	842 (678–1009) ($n=10$)	731 (602–1099) ($n=8$)	0.59
CRP, mg/dl	0.63 (0.10–1.96)	1.06 (0.17–2.16)	0.7
Ferritin, ng/ml	409 (248–843)	1600 (835–1935)	0.017
IL-18, pg/ml (normal range 18–121)	503 (343–727) ($n=10$)	540 (338–798) ($n=7$)	0.70
Anti-MDA5ab, U/ml	129.3 (44.6–254.0)	332.1 (92.0–599.8)	0.099
Treatment			
PSL + IVCY + CNI	5 (46)	7 (78)	0.2
PSL ± IVCY or CNI	6 (54)	2 (22)	
Improvement of ILD	11 (100)	0 (0)	<0.0001

The age values are presented as the mean (S.D.), and laboratory markers and pulmonary function tests are presented as the median (interquartile range).

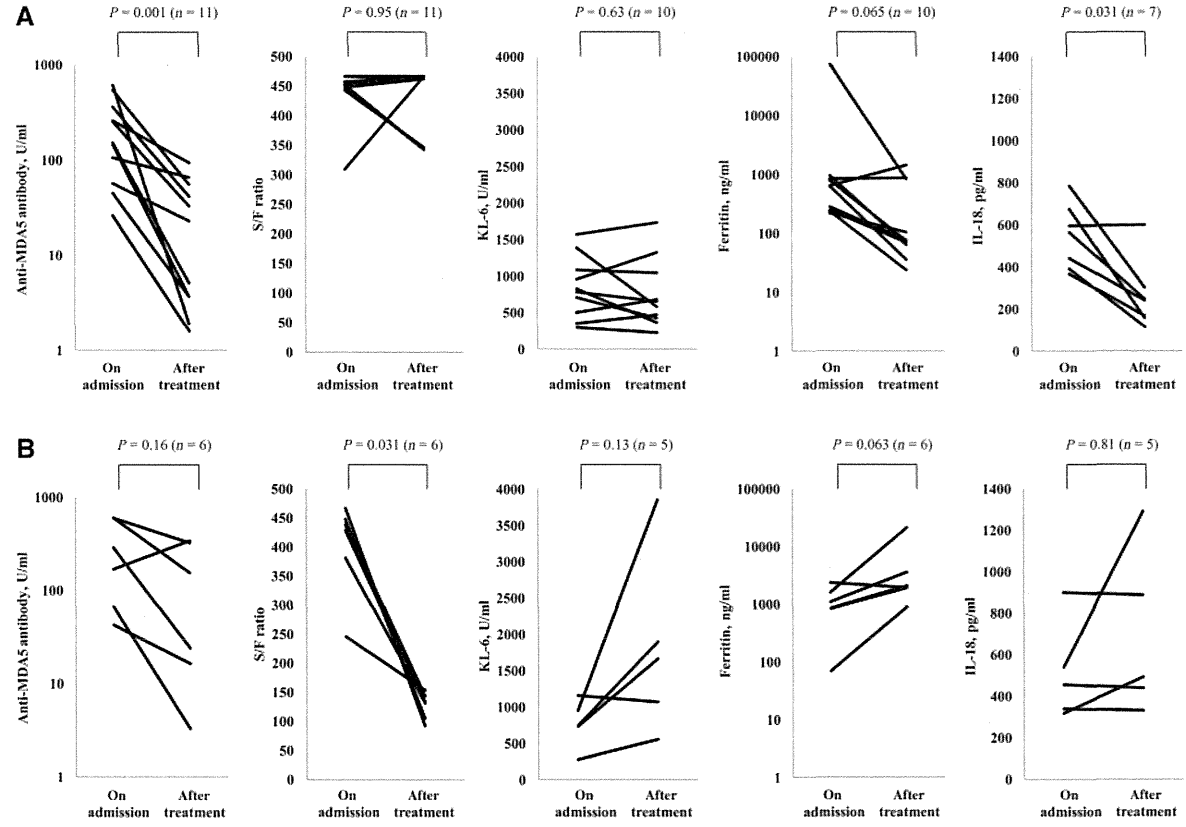
refractory RP-ILD and died within 6 months of treatment because of respiratory failure resulting from RP-ILD. We compared the clinical parameters upon admission with the parameters after treatment in each subset (Fig. 2). The median duration of evaluation after treatment was 3 months in the living subset and 2 months in the dead subset. There was no significant difference ($P=0.21$) between the two subsets in terms of the duration of evaluation after treatment. The data for each clinical parameter could only be partially obtained in some patients. The number of patients for whom data were obtained is indicated in each figure panel.

FIG. 1 Cumulative 100-month survival rates for all patients with anti-MDA5ab-positive DM (**A**), and the RP-ILD and non-RP-ILD subsets of anti-MDA5ab-positive DM patients (**B**).



The cumulative 100-month survival rates were calculated using the Kaplan–Meier test. The log-rank test was also used to compare survival rates. Survival rates and *P*-values are indicated in each figure panel.

FIG. 2 Comparison between clinical parameters upon admission and after treatment in patients with anti-MDA5ab-positive DM.



Based on patient survival, we analysed clinical parameters in two subsets: the living subset and the dead subset (**A** and **B**). The number of patients for whom data were obtained is indicated in each figure panel. Statistical analyses were performed with the Wilcoxon signed-rank test for comparisons of median values.

Anti-MDA5ab titre was significantly lower ($P = 0.001$) after treatment than on admission in the living subset (Fig. 2A). Anti-MDA5ab disappeared after treatment in 5 (45%) of the 11 living patients. On the other hand, there was no statistically significant difference ($P = 0.16$) in the dead subset between the anti-MDA5ab titre upon admission compared with the antibody titre after treatment (Fig. 2B). Anti-MDA5ab was still present after treatment in all dead patients except one. Moreover, the decline index of the anti-MDA5ab titre after treatment was analysed and compared among each subset. The decline index of the anti-MDA5ab was calculated as follows: (the antibody titre after treatment—the antibody titre upon admission) × 100/(the antibody titre upon admission). The median decline indices of the anti-MDA5ab titre (interquartile range) were 90% (63–97%) and 68% (9–92%) in the living and dead subsets, respectively.

In the dead subset, the S/F ratio was significantly lower after treatment ($P = 0.031$). The levels of KL-6 tended to decrease in the living subset and increase in the dead subset. On the other hand, the levels of ferritin more sensitively reflected the response to treatment than the levels of KL-6. The median values of ferritin after treatment were 76 ng/ml and 1987 ng/ml in the living and dead subsets, respectively ($P = 0.0017$). Moreover, the levels of IL-18 were significantly lower ($P = 0.031$) after treatment in the living subset. In the dead subset, the levels of IL-18 were not significantly lower after treatment.

Discussion

We have measured the clinical characteristics of disease and have demonstrated an association between clinical parameters and clinical course in patients with anti-MDA5ab-positive DM. The clinical manifestations of anti-MDA5ab-positive DM have been reported, mainly in Japanese studies [7–10]. Two different subsets of ILD with CADM patients are those with RP-ILD or with chronic ILD [19]. Fathi *et al.* [20] have reported that patients with inflammatory myopathy with ILD require careful evaluation of their clinical features because the course of ILD cannot be predicted at the first examination. However, we determined that investigation of both the anti-MDA5ab and the serum ferritin concentration are useful for predicting the onset of RP-ILD in DM [7, 21]. On the other hand, the serum ferritin level was <500 ng/ml in some patients with DM-associated RP-ILD [12]. These patients with RP-ILD were occasionally positive for the anti-aminoacyl-tRNA synthetase antibody and appeared to be well controlled with CSs and immunosuppressant agents compared with patients with DM-associated RP-ILD having anti-MDA5ab and/or hyperferritinaemia. This distinction in response to treatments might be responsible for the cellular phenotypes affecting the pathogenesis of ILD. Taken together, if the serum ferritin level is high in patients with DM, it should be considered that these patients may have anti-MDA5ab, and their clinical course may be complicated by RP-ILD.

CADM with RP-ILD showed a rapidly progressive pattern with a 6-month survival rate of 40.8–45%, which corresponded to the results in our study [22, 23]. In our study, $AaDO_2$ levels ⩾32 mmHg and ferritin levels ⩾828 ng/ml on admission were poor prognosis factors for RP-ILD with anti-MDA5ab-positive DM. The median anti-MDA5ab titre on admission was higher in the patients who died than in the living patients, although the difference between the two subsets was not statistically significant. However, the median anti-MDA5ab titre on admission was higher in the patients without RP-ILD than in those with RP-ILD. The anti-MDA5ab titre before treatment was not predictive of the prognosis of RP-ILD in anti-MDA5ab-positive DM. Measuring levels of serum ferritin and $AaDO_2$ before treatment is useful for predicting the prognosis of RP-ILD in DM.

We analysed the association between the anti-MDA5ab titre and the clinical course. We confirmed that the anti-MDA5ab titre has disappeared in improving surviving patients in our longitudinal observation (data not shown). Relapse has not occurred in any of the improving surviving patients except one. In the future we will investigate whether the anti-MDA5ab titre is increased again in either a pulmonary flare or skin exacerbation. Moreover, we have analysed several patients, in whom the serum ferritin level and IL-18 level were high, and were correlated with the clinical course in patients with RP-ILD with DM [24]. Immunosuppressive therapy had some effect on clinical parameters such as cytokines and antibodies regardless of clinical course. In the present study, however, immunosuppressive therapy was received more intensively in the dead subset than in the living subset of patients with anti-MDA5ab-positive DM. The frequency of receiving PSL + CNI + IVCY was higher in the dead subset than in the living subset. Moreover, there was no significant difference between the two subsets in terms of the duration of evaluation after treatment. Taken together, the sustained high levels of anti-MDA5ab, ferritin and IL-18 could be attributed to the poor response to treatment in the dead subset. Investigations of the anti-MDA5ab titre, ferritin level and IL-18 level after treatment are useful for predicting the clinical course and evaluating the response to treatment in patients with ILD with anti-MDA5ab-positive DM.

The levels of serum ferritin and IL-18 were associated with the status of ILD with anti-MDA5ab in the present study, as shown in previous reports [9, 12, 24]. Serum ferritin is an important laboratory finding of MAS [14]. MAS is now an accepted term that is used to refer to a form of secondary haemophagocytic lymphohistiocytosis observed in the context of rheumatic disorders [14, 15]. The pathophysiology of MAS involves a lack of T lymphocyte regulation and the excessive production of cytokines, such as TNF-α, IL-1β, IL-6 and IL-18, resulting in the activation of macrophages [15, 25]. The mRNA for IL-18 and IL-12 is readily detected in Kupffer cells and activated macrophages, and dendritic cells produce IL-18 in active inflammatory myopathies [26, 27]. We also reported that IL-18 is a key mediator in ILD with DM [13]. Moreover,

alveolar macrophages activated by some antigens, microbes and autoimmune stimuli are induced to produce leukotriene B4 and IL-8. These factors stimulate neutrophils to induce fibrosis in the lungs [28]. The MDA5 protein initially recognizes picornaviruses, such as the Coxsackie virus, and evokes antiviral responses by producing type I IFNs and TNF-α [11]. Previously, Coxsackie virus infection was reported to be one of the contributing factors in the pathogenesis of JDM [29]. Anti-MDA5ab-positive DM may be a type of MAS mainly in the skin and lungs that contributes to infections such as those caused by the Coxsackie virus. In conclusion, anti-MDA5ab titre, serum ferritin and IL-18 are useful for the evaluation of the response to treatment of RP-ILD with anti-MDA5ab-positive DM.

Rheumatology key messages

- Anti-MDA5ab is a disease-specific marker in DM with RP-ILD.
- Anti-MDA5ab titre, ferritin and IL-18 are useful for evaluation of response to treatment in DM with RP-ILD.

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