## *Results*

Clinical characteristics and the course of CIMDs treated with risperidone in the present six patients with SLE are shown in Table 1. Corticosteroids were administrated at a mean dosage of 51.7 mg/d (range, 40–60 mg/d) as prednisolone. This was the peak dosage of corticosteroids in the course of CIMDs. New-onset mood disorders, accompanied by manic features in four patients and mixed features in two, developed in a mean of 12.7 days (range, 2–28 days) of corticosteroid administration.

The mean score of the Young Mania Rating Scale (YMRS)[17] at baseline (at the point that psychiatric consultants saw the patients and diagnosed CIMD, at which point a decision was made to start risperidone) was 28.2 points (range, 20–43 points). Risperidone was started at a mean of 1.8 mg/d (range, 1–4 mg/day) and was flexibly increased up to a mean of 3.0 mg/d (range, 1–9 mg/d). The YMRS score was decreased to a mean of 13.8 points (range, 6–18 points) in 1 week and 8.0 points (range, 0–14 points) in 2 weeks. During this period, the corticosteroid dosage was the same as that at baseline, except for case 4. Four weeks later, the YMRS score was further decreased to a mean of 2.7 points (range, 0–4 points). At this point, the tapering of corticosteroid dosage had already started in all patients. Subsequently, all CIMDs in these six patients were resolved completely during the reduction in corticosteroid dosage without additional immunosuppressive agents, and risperidone was discontinued. The mean dosage of corticosteroids at this time was 32.5 mg/d (range, 15–45 mg/d) given as prednisolone.

Cases 2 and 3, with mixed episodes, recovered via a major depressive episode. Case 2's mixed episode at baseline (YMRS 23; Hamilton Depression Rating Scale [HAM-D] 27) was ameliorated with risperidone in 56 days, but a severe major depressive episode developed (HAM-D 31). We used milnacipran, a serotonin-noradrenalin reuptake inhibitor, up to 100 mg/d, but it was not effective. Next, we switched to dosulepin (75 mg/d) with good results. She recovered in a total of 240 days. In case 3, the mixed episode at baseline (YMRS 30; HAM-D 28) was ameliorated with risperidone in 35 days but shifted to a mild major depressive episode (HAM-D 13) that improved gradually after the discontinuation of risperidone without using antidepressants. She recovered in a total of 90 days.

These six patients tolerated risperidone well, except for sedation in case 4 and mild Parkinsonism in case 5. Metabolic effects of risperidone in combination with corticosteroids are

**TABLE 2. Metabolic Effects of Risperidone Treatment in Combination with Corticosteroids**

| Case | Duration of RPD Treatment, Days | Body Weight, kg | | FPG, mg/dl | | LDL-C, mg/dl | | HDL-C, mg/dl | | TG, mg/dl | | Parallel Medication for | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Baseline | Post-Treatment | Baseline | Post-Treatment | Baseline | Post-Treat | Baseline | Post-Treat | Baseline | Post-Treatment | Diabetes | Dyslipidemia |
| 1 | 40 | 43.6 | 40.8 | 72 | 68 | 156* | 116 | 74 | 96 | 239* | 284* | No | Yes |
| 2 | 56 | 53.2 | 51.0 | 94 | 93 | 170* | 154* | 49 | 58 | 259* | 230* | No | Yes |
| 3 | 35 | 58.4 | 56.4 | 98 | 75 | 139 | 130 | 80 | 84 | 142 | 153* | No | No |
| 4 | 14 | 65.0 | 64.0 | 123* | 119* | 180* | 154* | 77 | 74 | 104 | 123 | No | No |
| 5 | 28 | 45.2 | 45.6 | 89 | 75 | 166* | 188* | 47 | 34* | 149 | 173* | No | Yes |
| 6 | 56 | 49.0 | 50.2 | 94 | 84 | 131 | 146* | 61 | 66 | 113 | 245* | No | No |

RPD = risperidone; FPG = fasting plasma glucose; LDL-C = low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol; TG = triglycerides.
* Abnormal; FPG ≥ 100 mg/dl; LDL-C ≥ 140 mg/dl; HDL-C < 40 mg/dl; TG ≥ 150 mg/dl.

shown in Table 2. Abnormalities of fasting plasma glucose (≥100 mg/dL) at baseline vs. post-risperidone-treatment were detected in 1 vs. 1 of the six patients; low-density lipoprotein (LDL) cholesterol (≥140 mg/dL) in 4 vs. 4; high-density lipoprotein cholesterol (<40 mg/dL) in 0 vs. 1; triglycerides (≥150 mg/dL) in 2 vs. 5. No patient took parallel medication for diabetes, while three patients (case 1, 2, and 5) took parallel medication for dyslipidemia during the risperidone treatment.

## *Discussion*

In five of six patients in the present case series, the effectiveness of risperidone alone in the first 2 weeks was evident because the corticosteroids dosage was fixed in this period. In the following periods, recovery from CIMDs mirrored the tapering of the corticosteroids dosage, but risperidone may have assisted the recovery, although we could not clearly differentiate effects between the two.

As for adverse effects of risperidone treatment, sedation occurred in one patient, who had impaired renal function (serum creatinine, 1.19 mg/dL) caused by lupus nephritis. With lupus nephritis, a careful dose setting is required because the clearance of the active metabolite of risperidone is reduced in patients with renal diseases.[18] Parkinsonism occurred in one patient, when a high dose of risperidone was used (up to 9 mg/d), but it disappeared during the tapering of risperidone.

Combining an SGA such as risperidone with a corticosteroid could show an additive effect in terms of metabolic syndrome.[19] In our short-term observation, dyslipidemia, particularly abnormality of LDL cholesterol or triglycerides, was observed after the risperidone treatment in all cases, although the direct metabolic effect of risperidone could not be determined. Further controlled studies are necessary.

It is often difficult to distinguish CIMDs from CNS-SLE because no diagnostic gold standard for CNS-SLE exists[20] and because of the complicating fact that CNS-SLE manifestation can be triggered by corticosteroid therapy.[14] The present six patients could be diagnosed as definitely having CIMDs because of no findings suggesting CNS-SLE, as well as because of their complete recovery through a reduction in corticosteroid dosage without additional immunosuppressive agents. Strict definition of CIMDs should be required in the SLE population when extending the findings to other patient cohorts and medical groups.

Limitations of the present study include its small sample size, open-label design, and very specific cohort of patients: female Japanese, all having SLE. But our experience suggests risperidone is beneficial, especially in the first 2 weeks, in the treatment of CIMDs with manic or mixed features in patients with SLE, although the dosage reduction of corticosteroids contributes to the ultimate resolution of CIMDs. Further controlled studies are necessary.

Corticosteroids are the cornerstone of treatment for various inflammatory and immunologically mediated disorders, such as SLE. When CIMDs occur, corticosteroids cannot be rapidly tapered or discontinued in most cases given their risk/benefit rates. Our results may contribute to the guidance of clinicians with regard to the management of CIMDs in such cases.

*Disclosure: The authors report no financial or other relationship relevant to the subjects of this article. This case-series is, in part, from a subset of a cohort study presented at the 55th Annual Meeting of the Academy of Psychosomatic Medicine, Miami, Florida, November, 2008.*

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Original article

# Up-regulated expression of *HLA-DRB5* transcripts and high frequency of the *HLA-DRB5*01:05* allele in scleroderma patients with interstitial lung disease

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## Abstract

**Objective.** Interstitial lung disease (ILD) is a serious complication of SSc. We aimed to identify markers associated with SSc-related ILD.

**Methods.** RNA was prepared from the peripheral blood mononuclear cells of 14 SSc patients, divided into four different RNA pools according to the presence or absence of ILD and to the treatment, and subjected to microarray analysis. Real-time quantitative PCR was used to confirm the microarray results in 43 SSc patients, 42 autoimmune controls and 10 healthy controls. Genomic DNA samples were collected from 149 patients with SSc (70 in Hokkaido and 79 in Tokyo) who underwent a high-resolution CT for the evaluation of ILD and from 230 healthy controls. Genotyping was performed using sequence-specific primers.

**Results.** The microarray analysis revealed *HLA-DRB5* to be the only gene commonly up-regulated in patients with ILD compared with those without ILD in both comparison groups. High expression levels of *HLA-DRB5* in SSc patients with ILD were confirmed by real-time quantitative PCR. The prevalence of *HLA-DRB5* gene carriers increased in the SSc patients with ILD relative to those without ILD or to healthy controls in both cohorts. Among the four detected alleles, the *HLA-DRB5*01:05* allele was significantly more frequent in SSc patients with ILD than in SSc patients without ILD or in healthy controls. These associations were confirmed in the second cohort.

**Conclusion.** *HLA-DRB5* was highly expressed in PBMCs from patients with SSc-related ILD. The *HLA-DRB5*01:05* allele is a risk factor for ILD in patients with SSc.

**Key words:** SSC, human leucocyte antigen, pulmonary fibrosis, microarray, peripheral blood mononuclear cells, gene expression, risk factor.

BASIC

## Introduction

SSc is an autoimmune connective tissue disorder characterized by microvascular injury, skin fibrosis and distinctive visceral changes. SSc is a clinically heterogeneous disease, ranging from a mild form with less extensive involvement of the viscera to a more severe type with widespread visceral changes significantly affecting morbidity and mortality [1–4]. Interstitial lung disease (ILD), one of the most serious complications of SSc, develops in >50% of patients with SSc [5, 6]. In contrast to the recent improvements in the survival of patients with SSc complicated by renal crisis, the frequency of deaths due to ILD among SSc patients has increased significantly over the last 30 years, from 6 to 33% of SSc-related deaths [7]. Other studies have concurred that the presence of ILD is associated with a poorer prognosis in patients with SSc [8–10]. Although modest clinical efficacy of cyclophosphamide was reported in two high-quality randomized controlled trials [11, 12], and potentially effective treatments, such as mycophenolate mofetil,

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imatinib mesylate or haematopoietic stem cell transplantation (HSCT), remain to be evaluated [13, 14], the available treatments targeting SSc-related ILD are still limited. Therefore the search for novel biomarkers or genetic predispositions specific for SSc-related ILD is critical to provide new insights into the disease process, potentially leading in the longer term to a better prognosis for this severe visceral complication. Genetic backgrounds, up-regulated cytokines/growth factors and signal molecules such as HLA, *PTPN22*, *CTGF*, *TGF-β* and *PDGF* have been implicated in the pathophysiology of SSc [15–17]. However, until recently, few studies have focused on specific biomarkers in SSc-related ILD.

In the present study we aimed to identify up-regulated molecule(s) in patients with SSc-related ILD based on an unbiased microarray analysis using pooled RNA samples. The analysis of four pools of RNA derived from peripheral blood mononuclear cells (PBMCs) identified *HLA-DRB5* as a candidate up-regulated gene in patients with ILD. We then confirmed its expression in individual patients and also genotyped the *HLA-DRB5* gene in two cohorts. Detailed genotyping led us to identify the specific *HLA-DRB5* allele **01:05* as a novel risk factor for SSc-related ILD.

## Patients and methods

This study was approved by the ethical committees of the Hokkaido University Graduate School of Medicine and the Tokyo Women's Medical University. Informed patient consent was obtained for this study.

### Global gene expression analysis using DNA microarray

We first evaluated global gene expression profiles in pooled RNA from the PBMCs of patients with SSc. Four sets of RNA pools were prepared from PBMCs from 14 SSc patients seen at the Hokkaido University Hospital. The first pool was prepared from four patients with ILD [three females, median age 46 (30–58) years, median disease duration 84 (70–119) months] who received only conventional therapies [prostaglandin and/or the following immunosuppressive agents: prednisolone ($n=1$), ciclosporin ($n=1$), tacrolimus ($n=1$) and CYC ($n=1$)]. The second pool was derived from four patients without ILD [three females, median age 60 (40–64) years, median disease duration 102 (60–234) months] who received conventional therapies [prostaglandin and/or immunosuppressive agents: ciclosporin ($n=1$)]. The third pool included four patients with ILD [three females, median age 39 (22–61) years, median disease duration 93 (66–103) months] who received HSCT. The last array comprised two patients without ILD [one female, median age 49 (33–65) years, median disease duration 93 (54–132) months] who received HSCT. The mixed RNA samples in each pool were subjected to a single DNA microarray (Affymetrix GeneChip Human Gene 1.0 ST Array, Affymetrix, Santa Clara, CA, USA). Data were analysed using GeneSpring GX version 10.0 (Agilent Technologies, Inc., Santa Clara, CA, USA). The fluorescence intensity results were defined as RAW normalized according to the Robust Multi-array Average algorithm. To normalize the treatment background of the patients, the first comparison performed was with the conventional therapy group, and the second was with the HSCT group. Up-regulated genes were defined as those for which the gene expression in the first or the third pool increased more than 3-fold relative to that in the second or the fourth pool, respectively.

### Quantitative real-time PCR

All patients were observed at the Hokkaido University Hospital. Our subjects comprised 43 SSc patients {25 with ILD [19 females, median age 55 (18–77) years, median disease duration 74 (9–282) months] and 18 without ILD [15 females, median age 59 (28–83) years, median disease duration 84 (4–345) months]}. Among the patients with ILD, 5 were treated by HSCT and 20 were on immunosuppressive agents [prednisolone ($n=10$), ciclosporin ($n=6$), tacrolimus ($n=4$), CYC ($n=2$) and D-Pen ($n=3$)]. Among the patients without ILD, 3 were treated by HSCT, and 15 were on prostaglandin and/or immunosuppressive agents [prednisolone ($n=6$), ciclosporin ($n=2$) and D-Pen ($n=5$)]. As control groups, PBMCs were also collected from 42 patients with other autoimmune diseases [RA ($n=20$ and DM/PM ($n=22$)] and from 10 healthy controls. The expression levels of *HLA-DRB5* and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in each individual were evaluated by real-time quantitative PCR using TaqMan Gene Expression assays (*HLA-DRB5* assay ID: Hs03046116_m1, GAPDH assay ID: Hs9999905_m1), TaqMan Universal PCR Master Mix and the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). Relative quantification was performed using the comparable cycle threshold (CT) method in which ΔCT is defined as the level of the HLA-DRB5 transcript in the RNA sample relative to that of the GAPDH transcript, as previously reported [18].

### Genomic DNA extraction and genotyping of *HLA-DRB5* and *HLA-DRB1*

The first cohort comprised 70 Japanese patients with SSc who visited the Hokkaido University Hospital and fulfilled the 1980 criteria of the ACR [19] and 147 disease-free Japanese volunteers residing in the Hokkaido area as healthy controls. Patients with SSc were classified as either having ILD ($n=41$) or not having ILD ($n=29$) based on HRCT, as described in the next subsection. Of the 41 patients with ILD, 6 were treated with HSCT using autologous peripheral blood stem cells, and the other 35 patients received conventional therapy. HSCT was performed as previously described [20]. Of the six patients who received HSCT, three also received ciclosporin for recurrent or progressive scleroderma. Conventional therapy included prostaglandin and immunosuppressive agents [prednisolone ($n=16$), ciclosporin ($n=5$), tacrolimus ($n=7$), CYC ($n=2$) and/or D-Pen ($n=5$)]. Of the 29 patients without ILD, 4 were treated with HSCT and the other 25 with conventional therapy. Patients with other autoimmune diseases comprised 20 with RA and

29 with DM or PM. RA patients fulfilled the ACR criteria [21], and DM/PM patients fulfilled the Bohan and Peter criteria [22, 23]. The second cohort comprised 83 disease-free Japanese volunteers residing in the Tokyo area and 79 Japanese patients with SSc observed at the Tokyo Women's Medical University Hospital. These patients with SSc were classified as having ILD ($n = 40$) or as not having ILD ($n = 39$) (Table 1).

Genomic DNA samples were extracted using DNA Quick II (DS Pharma Biomedical Co. Ltd, Osaka, Japan), according to the manufacturer's instructions. *HLA-DRB5* genotyping was performed by PCR with sequence-specific primers (PCR-SSP) using Olerup SSP *HLA-DRB5* (Olerup SSP AB, Saltsjöbaden, Sweden). All of the 19 known alleles of *HLA-DRB5* are theoretically identifiable using this PCR-SSP method [24, 25]. The *HLA-DRB1* alleles were determined using the PCR-SSOP Luminex method with LABType SSO (One Lambda Inc., Canoga Park, CA, USA), a reverse SSO DNA typing system, according to the manufacturer's instructions.

### Evaluation of pulmonary and other organ involvement

The presence or absence of ILD was evaluated using HRCT in all of our patients with SSc. The diagnosis of ILD was made when one or more of the following features were evident: isolated ground-glass opacities, honeycombing, ground-glass attenuation and/or traction bronchiectasis. After the diagnosis of ILD was made, the HRCT findings were classified into the following: non-specific interstitial pneumonia (NSIP), usual interstitial pneumonia (UIP), or organizing pneumonia (OP). Pulmonary hypertension (PH) was screened for by echocardiography and confirmed by right-heart catheterization [26]. Other organ involvement was defined as follows: renal (renal crisis in patients with SSc and nephritis in other autoimmune diseases), cardiac (arrhythmia), joint (arthritis) and muscle (continuous increase in serum creatinine kinase and/or serum aldolase levels, electromyogram and/or muscle biopsy).

### Autoantibodies

ANA was detected with IIF. A titre of 1:80 or more was considered to be positive. Anti-topo-I antibody (anti-Topo-I), ACA, anti-U1-RNP antibody and anti-SS-A antibody levels were examined using enzyme-linked immunoassays (MESACUP, Medical & Biological Laboratories Co., Ltd, Japan), RF levels were evaluated by the latex turbidimetric assay (N-assay TIA RF Nittobo, Nittobo

**Table 1** Clinical features of the patients with SSc and the autoimmune controls

| Characteristics | | First cohort | | | Second cohort |
|---|---|---|---|---|---|
| | | SSc ($n = 70$) | RA ($n = 20$) | DM/PM ($n = 29$) | SSc ($n = 79$) |
| Gender (female/male) | | 55/15 | 16/4 | 22/7 | 73/6 |
| Age, mean (S.D.), years | | 51.0 (13.6) | 54.4 (13.8) | 52.2 (14.2) | 41.0 (12) |
| Disease duration, median (range), months | | 26.0 (2–516) | 86.0 (4–408) | 24.0 (1–380) | ND |
| Smokers, *n* | | 28 | 10 | 9 | ND |
| Diffuse/limited | | 45/25 | | | 48/31 |
| Organ involvement, *n* (%) | | | | | |
| ILD | | 41 (58.6) | 7 (35) | 17 (58.6) | 40 (50.6) |
| HRCT findings | | | | | |
| NSIP | | 25 | 5 | 15 | ND |
| UIP | | 16 | 1 | 0 | ND |
| OP | | 0 | 1 | 2 | ND |
| PH | | 11 (15.7) | 0 (0) | 1 (3.4) | 8 (10.1) |
| Digital ulcer | | 6 (8.6) | 0 (0) | 0 (0) | 14 (17.7) |
| Renal | | 7 (10.0) | 0 (0) | 3 (10.3) | 0 (0) |
| Cardiac | | 7 (10.0) | 0 (0) | 8 (27.6) | ND |
| Joint | | 9 (12.9) | 20 (100) | 5 (17.2) | ND |
| Muscle | | 2 (2.9) | 1 (5.0) | 29 (100) | ND |
| Autoantibodies, *n* (%) | | | | | |
| ANA | ⩾1:80 | 69 (98.6) | 16 (80.0) | 19 (65.5) | 79 (100) |
| aTopo-I | >18.9 INDEX | 22 (31.4) | NA | NA | 29 (36.7) |
| ACA | >15.9 INDEX | 18 (25.7) | NA | NA | 16 (20.2) |
| aU1-RNP | >12.9 INDEX | 11 (15.7) | NA | NA | NA |
| RF | >16.3 U/L | 7 (10.0) | 16 (80.0) | 8 (27.6) | NA |
| aJo-1 | >12.9 INDEX | 0 (0) | NA | 8 (27.6) | NA |
| aSS-A/Ro | >9.9 INDEX | 17 (24.3) | NA | NA | NA |
| aDNA | >6 U/L | 4 (5.7) | NA | NA | NA |

Organ involvement was defined as follows: lung (interstitial pneumonia as shown by HRCT, with findings based on radiologists' interpretation), renal (renal crisis in patients with SSc and nephritis in patients with other diseases), cardiac (arrhythmia), joint (arthritis), muscle (continuously increased serum creatinine kinase or serum aldolase, or both). aTopo-I: anti-topoisomerase-I; aU1-RNP: anti-U1-RNP antibody; aSS-A/Ro: anti-SS-A antibody; NA: not available; ND: no data.

Medical Co. Ltd, Tokyo, Japan). Anti-DNA (aDNA) antibody levels were measured using radioimmunoassay (SRL, Inc., Japan).

### Isolation of PBMCs and RNA extraction

PBMCs were obtained from heparinized venous blood using gradient centrifugation over Ficoll-Paque PLUS (Amersham Biosciences Corp., NJ, USA). Total RNA levels from PBMCs were isolated using the RNeasy Mini Kit (QIAGEN Science, Germantown, MD, USA).

### Statistical analysis

Calculations were performed using the statistical software package SPSS statistics (version 19.0). Comparisons of mRNA expression in PBMCs were performed using the Mann–Whitney test. Univariate analyses were performed using the chi-squared test and Fisher's exact test. When a value was zero, this number was replaced by 0.5 to perform the chi-squared test. With regard to multiplicity, the Bonferroni correction was used to adjust the significance levels for the analysis of frequencies of the *HLA-DRB5* and *HLA-DRB1* alleles. Other analyses, namely the analysis of the prevalence of carriers of *HLA-DRB5* and the comparisons of demographic and clinical parameters of SSc patients with and without *HLA-DRB5*01:05*, were regarded as exploratory, and therefore no corrections for multiple testing were performed. The demographic variables, clinical independent variables and genetic risk factors were evaluated for their associations with ILD in multivariate logistic regression analyses.

## Results

### Evaluation of global gene expression

In the patients who received HSCT, two genes were up-regulated in patients with ILD relative to those without ILD. In the conventional treatment group, 17 genes were up-regulated in patients with ILD compared with those without ILD. Of these genes, *HLA-DRB5* was the only commonly up-regulated gene in both comparisons (Table 2).

### Quantitative real-time PCR

*HLA-DRB5* gene expression levels were higher in the PBMCs from SSc patients with ILD than in those patients without ILD (Fig. 1). *HLA-DRB5* expression levels were related neither to disease duration nor to the presence or absence of immunotherapy (data not shown). In the relatively small numbers of patients with other autoimmune disease, there were apparently no differences in the levels of *HLA-DRB5* gene expression between patients with ILD and those without ILD or healthy controls.

### *HLA-DRB5* genotyping (the first cohort)

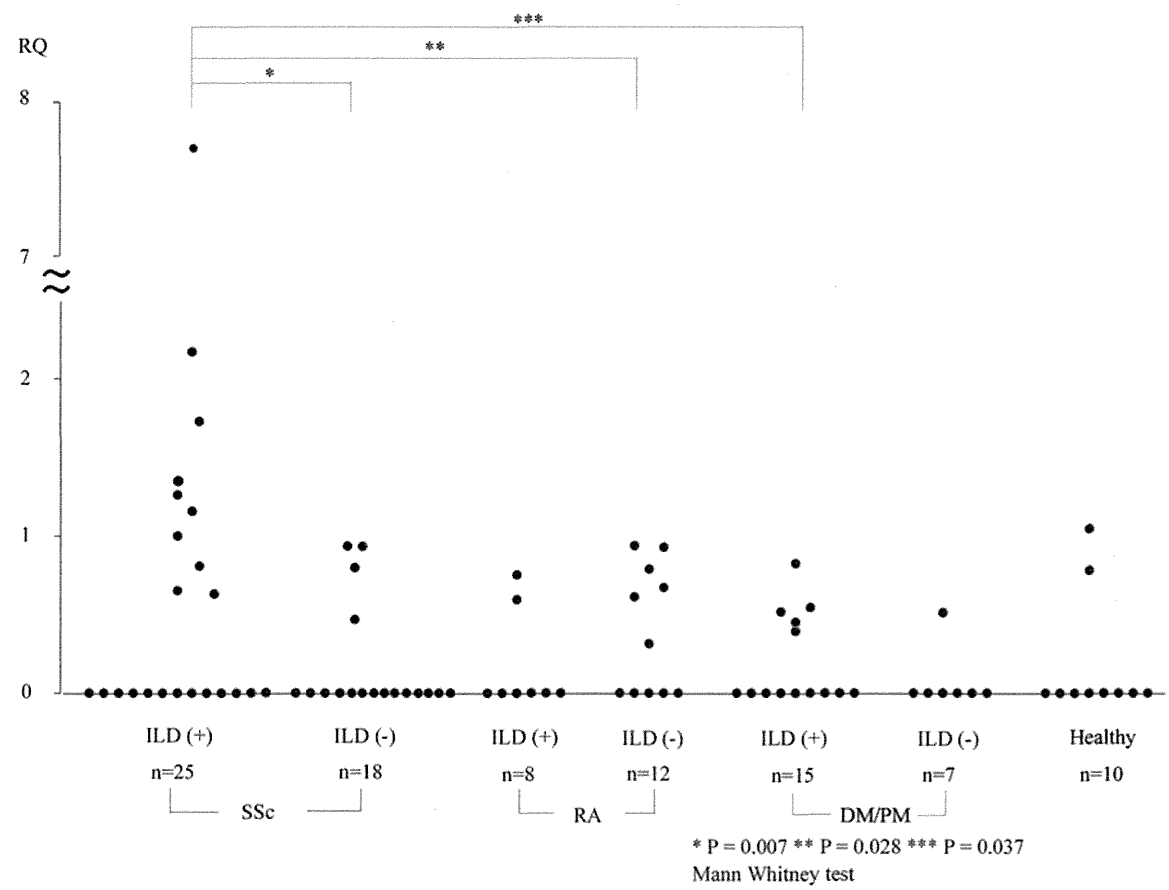
The prevalence of *HLA-DRB5* gene carriers among SSc patients was not significantly different from that in healthy controls. The prevalence of *HLA-DRB5* gene carriers in patients with RA or DM/PM was also not different from

**Table 2** Up-regulated genes in PBMCs from SSc patients with ILD as evaluated by DNA microarray

| Classification | Up-regulated genes | GenBank accession number | RAW data | | Ratio (fold increase) |
|---|---|---|---|---|---|
| | | | ILD$^+$ | ILD$^-$ | |
| Conventional therapy group | | | | | |
| Human lymphocyte antigen | *HLADRB5* | M20429 | 652.54 | 202.01 | 3.23 |
| Membrane receptors | IL8RA | L19591 | 1502.38 | 345.52 | 4.35 |
| | IL8RB | L19593 | 1405.91 | 351.07 | 4.00 |
| | FFAR2 | BC096198 | 697.9 | 214.9 | 3.25 |
| Signalling intermediates | CYP4F3 | AB002454 | 746.32 | 150.11 | 4.97 |
| | PTGS2 | AY151286 | 1381.82 | 324.75 | 4.25 |
| | MME | J03779 | 724.01 | 171.75 | 4.22 |
| | PROK2 | AF333025 | 1186.54 | 289.64 | 4.10 |
| | KCNJ15 | BC013327 | 285.81 | 89.85 | 3.18 |
| | ALPL | BC090861 | 473.19 | 152.16 | 3.11 |
| | CNTNAP3B | AL353791 | 102.19 | 33.30 | 3.05 |
| | MGAM | AF016833 | 421.15 | 138.45 | 3.04 |
| Translation initiation factor | EIF1AY | AF000987 | 331.90 | 81.58 | 4.07 |
| snRNA | SNOAD13 | X58062 | 4821.71 | 1184.11 | 4.07 |
| | RNU5E | M77839 | 2884.99 | 759.51 | 3.80 |
| | SNOAD60 | | 605.15 | 193.12 | 3.13 |
| | SNOAD41 | X96640 | 866.85 | 283.01 | 3.06 |
| HSCT group | | | | | |
| Human lymphocyte antigen | *HLADRB5* | M20429 | 1083.57 | 298.12 | 3.63 |
| Signalling intermediates | ERAP2 | AB163917 | 999.11 | 259.17 | 3.86 |

snRNA: small nuclear ribonucleic acid.

FIG. 1 Quantitative evaluation of *HLA-DRB5* transcripts in PBMCs.




PBMCs collected from 43 SSc patients (25 with ILD and18 without ILD), 42 patients with other autoimmune diseases [RA ($n = 20$), DM/PM ($n = 22$)] and 10 healthy controls were evaluated for the levels of *HLA-DRB5* transcripts using real-time qPCR. Relative quantification was performed using the $C_T$ method, in which $\Delta C_T$ is defined as the level of *HLA-DRB5* transcript in the RNA sample relative to that of the GAPDH transcript.

that in healthy controls. However, the prevalence of *HLA-DRB5* gene carriers was higher in SSc patients with ILD than in those without ILD or in healthy controls. In contrast, *HLA-DRB5* was not associated with ILD in patients with RA or DM/PM, although the sample size for our autoimmune control group was relatively small (Table 3). In patients with SSc, *HLA-DRB5* was not associated with other clinical complications such as PH, renal crisis or digital ulcer in patients with SSc or in the presence or absence of the evaluated autoantibodies (data not shown).

Genotyping of the *HLA-DRB5* gene revealed 4 of the known 19 alleles in the SSc patients and healthy controls: **01:01*, **01:02*, **01:05* and **02:02*. Among the different *HLA-DRB5* alleles, the *HLA-DRB5*01:05* allele was significantly more frequent in SSc patients than in the controls. More specifically, *DRB5*01:05* was significantly more frequent in SSc patients with ILD, but not in SSc patients without ILD, compared with healthy controls (Table 4). In addition, the *DRB5*01:05* allele was more frequent in SSc patients with ILD than in those without ILD.

In the univariate analysis, the presence of the *DRB5*01:05* allele was associated with PH. However, no other clinical features, including age, gender, disease duration, renal crisis and all of the tested autoantibodies, were related to the presence of the *DRB5*01:05* allele (Table 5). The *DRB5*01:05* allele was not associated with ILD in patients with RA or PM/DM (data not shown).

### *HLA-DRB1* genotyping (the first cohort)

The frequencies of the *HLA-DRB1* alleles in the 70 SSc patients from the first cohort are shown in supplementary data Table 1, available as supplementary data at *Rheumatology* Online. A total of 20 *HLA-DRB1* alleles were identified. Of these alleles, the *HLA-DRB1**15:02 allele was more frequent in SSc patients with anti-Topo-I (50.0%) than in those patients without anti-Topo-I (20.8%, $P = 0.013$) but was not significantly increased in

**Table 3** Prevalence (%) of *HLA-DRB5* carriers among patients with SSc, RA, DM/PM and in healthy controls

| Patient group | *n* | Prevalence (%) | OR (95% CI) *vs* control | OR (95% CI) *vs* ILD (−) |
|---|---|---|---|---|
| First cohort | | | | |
| SSc | | | | |
| Total | 70 | 41.4 | 1.66 (0.92, 2.99) | 3.30** (1.16, 9.41) |
| ILD $^{+}$ | 42 | 52.4 | 2.58* (1.28, 5.19) | |
| ILD $^{-}$ | 28 | 25.0 | 0.78 (0.31, 1.97) | |
| RA | | | | |
| Total | 20 | 30.0 | 1.00 (0.36, 2.78) | 0.90 (0.12, 6.78) |
| ILD $^{+}$ | 7 | 28.5 | 0.94 (0.17, 5.01) | |
| ILD $^{-}$ | 13 | 30.8 | 1.04 (0.30, 3.56) | |
| DM/PM | | | | |
| Total | 29 | 34.6 | 1.05 (0.44, 2.50) | 0.83 (0.17, 4.09) |
| ILD + | 17 | 29.4 | 0.98 (0.32, 2.93) | |
| ILD − | 12 | 33.3 | 1.17 (0.33, 4.09) | |
| Controls | 147 | 29.9 | | |
| Second cohort | | | | |
| SSc | | | | |
| Total | 79 | 43.0 | 1.33 (0.71, 2.51) | 2.75**** (1.09, 6.92) |
| ILD + | 40 | 55.0 | 2.16*** (1.00, 4.65) | |
| ILD − | 39 | 23.4 | 0.79 (0.35, 1.77) | |
| Controls | 83 | 36.1 | | |

*$P = 0.012$, **$P = 0.022$, ***$P = 0.047$, ****$P = 0.030$. The chi-squared test or Fisher's exact test was employed. OR: odds ratio.

SSc patients with ILD relative to those without ILD. The *HLA-DRB1*04:03* and *DRB1*08:02* alleles were less frequent in SSc patients with ILD than in those without ILD. The frequencies of the other *HLA-DRB1* alleles did not significantly differ between SSc patients with and without ILD.

### Multivariate analysis (the first cohort)

In a multivariate analysis composed of clinical and demographic parameters after the inclusion of the HLA alleles, we identified *HLA-DRB5*01:05* and anti-Topo-I as independent risk factors for ILD ($P = 0.024$ and 0.011, respectively). Other parameters, such as *HLA-DRB1*15:02* or other *DRB5* alleles, were not significantly associated with ILD (supplementary data Table 2, available as supplementary data at *Rheumatology* Online).

### *HLA-DRB5* genotyping (the second cohort)

The prevalence of *HLA-DRB5* gene carriers in SSc patients was not significantly different from that in healthy controls. The prevalence of *HLA-DRB5* gene carriers was higher in SSc patients with ILD than in those without ILD or in healthy controls (Table 2). Neither clinical complications, such as PH, renal crisis or digital ulcer, nor measured autoantibody levels were associated with the *HLA-DRB5* allele (data not shown).

No association was found between SSc and the *DRB5*01:05* allele in the second cohort. The *HLA-DRB5*01:05* allele was significantly more frequent in patients with ILD than in healthy controls (Table 4). Thus the associations between SSc-related ILD and the *DRB5*01:05* allele were confirmed in the second cohort.

On univariate analysis, no clinical factors except ILD correlated with the presence of the *DRB5*01:05* allele (Table 5).

## Discussion

The *HLA-DRB5* locus and its alleles have rarely been brought to researchers' attention in terms of disease susceptibility. Taking advantage of unbiased mRNA expression profiling using pooled PBMC samples from SSc patients with or without ILD, we focused on *HLA-DRB5* and confirmed the high expression of this gene in individual patients with SSc-related ILD compared with those without ILD. Furthermore, we identified the *HLA-DRB5*01:05* allele as a novel risk factor for ILD in SSc using a genotyping approach. Due to the limitations of our cohort in terms of relatively small size and uniform ethnicity, our results clearly need to be confirmed in a larger cohort or in other ethnicities. As many of the RNA samples were obtained from patients currently under treatment, prospective observation would also be desirable to determine whether *HLA-DRB5* expression in PBMCs or the *HLA-DRB5*01:05* allele has predictive value for the development of ILD in patients with early-stage SSc. However, because *HLA-DRB5* expression is genetically regulated and is unaffected by disease duration or immunotherapy, it is highly unlikely that there is any significant discrepancy between *HLA-DRB5* expression levels and genotype.

The first genome-wide association study concerning SSc in a sample of European ancestry confirmed the role of *HLA* gene regions as SSc genetic risk factors

TABLE 4 Frequency (%) of *HLA-DRB5* alleles in SSc patients with and without ILD and in healthy controls

| Patient group | *n* | Frequency (%) | OR (95% CI) *vs* controls | OR (95% CI) *vs* ILD (–) |
|---|---|---|---|---|
| First cohort | | | | |
| *B5*01* | | | | |
| **01:01* | | | | |
| SSc total | 70 | 12.9 | 0.89 (0.38, 2.05) | 0.52 (0.13, 2.13) |
| ILD + | 41 | 9.8 | 0.65 (0.21, 2.01) | |
| ILD – | 29 | 17.2 | 1.25 (0.43, 3.64) | |
| Controls | 147 | 14.3 | | |
| **01:02* | | | | |
| SSc total | 70 | 18.6 | 1.30 (0.61, 2.75) | 2.44 (0.60, 9.94) |
| ILD + | 41 | 22.0 | 1.83 (0.79, 4.27) | |
| ILD – | 29 | 10.3 | 0.66 (0.18, 2.35) | |
| Controls | 147 | 14.9 | | |
| **01:05* | | | | |
| SSc total | 70 | 22.9 | 5.15§ (2.08, 12.73) | 4.02‡ (1.03, 15.74) |
| ILD + | 41 | 31.7 | 8.07§ (3.06, 21.28) | |
| ILD – | 29 | 10.3 | 2.00 (0.50, 8.06) | |
| Controls | 147 | 5.4 | | |
| *B5*02* | | | | |
| **02:02* | | | | |
| SSc total | 70 | 4.3 | 2.15 (0.42, 10.93) | |
| ILD + | 41 | 4.9 | 2.46 (0.40, 15.25) | |
| ILD – | 29 | 0 | 1.43 (0.07, 28.41) | |
| Controls | 147 | 2.0 | | |
| Second cohort | | | | |
| *B5*01* | | | | |
| **01:01* | | | | |
| SSc total | 79 | 10.3 | 0.74 (0.28, 1.94) | 0.53 (0.14, 2.02) |
| ILD + | 40 | 7.7 | 0.53 (0.14, 2.02) | |
| ILD – | 39 | 12.8 | 0.96 (0.31, 2.99) | |
| Controls | 83 | 14.3 | | |
| **01:02* | | | | |
| SSc total | 79 | 29.1 | 1.48 (0.73, 3.03) | 3.08 (1.08, 8.57) |
| ILD + | 40 | 40.0 | 2.41 (1.06, 5.47) | |
| ILD – | 39 | 17.9 | 0.79 (0.30, 2.08) | |
| Controls | 83 | 21.7 | | |
| **01:05* | | | | |
| SSc total | 79 | 8.9 | 7.97 (0.96, 66.36) | |
| ILD + | 40 | 17.5 | 17.39† (2.06, 146.94) | |
| ILD – | 39 | 0 | 1.44 (0.06, 36.06) | |
| Controls | 83 | 1.2 | | |

§$P < 0.001$, †$P = 0.009$, ‡$P = 0.036$. The chi-squared test or Fisher's exact test was used. *P*-values were adjusted using the Bonferroni correction. OR: odds ratio.

with the strongest association [27]. Previously, HLA studies in SSc have suggested that MHC genes exert their influence through the presentation of a specific self-antigen [28]. In the Japanese population, the frequencies of *DRB1*15:02*, *DQB1*06:01* and *DPB1*09:01* have been reported to be significantly increased in anti-Topo-I-positive patients [29]. In terms of pulmonary complications, an association between *DR3/DR52a* and pulmonary fibrosis has been documented in Caucasian patients with SSc [30]. However, *HLA-DR3* is quite rare in the Japanese population, and, in fact, none of the investigated individuals in our first cohort had a *DRB1* allele corresponding to this serotype. The correlation between the *HLA-DRB1*15:02* allele and anti-Topo-I antibodies was confirmed in our study, as well as the absence of a significant correlation between this allele and SSc or SSc-related ILD.

*HLA-DRB5* belongs to the HLA class II beta chain paralogues. Currently 19 alleles have been identified for the *HLA-DRB5* gene, and several studies have reported the frequencies of *HLA-DRB5* alleles and the *DRB5-DRB1-DQB1* haplotype in healthy and diseased populations [31–33]. However, no report has referred to the frequencies of the *HLA-DRB5*01:05* allele or the *DRB5*01:05-DRB1* haplotype. Although the frequency of the *HLA-DRB5*01:05* allele in the healthy Japanese population

**Table 5** Comparisons of demographic and clinical parameters between SSc patients with and without *HLA-DRB5*01:05*

| | | *HLA-DRB5 *01:05* | | OR (95% CI) |
|---|---|---|---|---|
| First cohort | | (+) $n = 16$ | (−) $n = 54$ | |
| Profile | *n* (%) | | | |
| Gender (male) | | 3 (18.7) | 11 (20.3) | 0.90 (0.22, 3.73) |
| Smokers | | 7 (43.8) | 21 (38.9) | 1.15 (0.37, 3.56) |
| Diffuse type | | 12 (75.0) | 33 (61.1) | 1.91 (0.54, 6.71) |
| Complication | | | | |
| ILD | | 13 (81.2) | 28 (51.9) | 4.02* (1.03, 15.74) |
| PH | | 5 (31.2) | 4 (7.4) | 5.56** (1.24, 24.91) |
| SRC | | 0 (0) | 2 (3.7) | |
| Digital ulcer | | 2 (12.5) | 6 (11.1) | 1.14 (0.21, 6.30) |
| Antibodies | | | | |
| ACA | | 4 (25.0) | 14 (25.9) | 0.95 (0.26, 3.44) |
| aTopo-I | | 6 (37.5) | 16 (29.6) | 1.43 (0.44, 4.58) |
| anti-U1-RNP | | 1 (6.25) | 10 (18.6) | 0.29 (0.03, 2.49) |
| RF | | 2 (12.5) | 5 (11.4) | 1.11 (0.19, 6.41) |
| aSS-A/Ro | | 1 (7.1) | 16 (34.8) | 0.14 (0.02, 1.20) |
| aDNA | | 0 (0) | 4 (9.3) | |
| Second cohort | | (+) $n = 7$ | (−) $n = 72$ | |
| Profile | *n* (%) | | | |
| Gender (male) | | 0 (0) | 6 (8.3) | |
| Diffuse type | | 6 (85.7) | 37 (51.4) | 5.68 (0.65, 49.56) |
| Complication | | | | |
| ILD | | 7 (100) | 0 (0) | *** |
| PH | | 1 (14.3) | 7 (9.7) | 1.55 (0.16, 14.77) |
| SRC | | 0 (0) | 0 (0) | |
| Digital ulcer | | 1 (14.3) | 13 (18.1) | 0.76 (0.08, 6.83) |
| Antibodies | | | | |
| ACA | | 1 (14.3) | 15 (20.8) | 0.63 (0.07, 5.67) |
| aTopo-I | | 4 (57.1) | 25 (34.7) | 2.51 (0.52, 12.09) |

*$P = 0.036$, **$P = 0.030$, ***$P < 0.001$. The chi-squared test or Fisher's exact test was used. OR: odds ratio; SRC: scleroderma renal crisis; aTopo-I: anti-topoisomerase-I antibody; aU1-RNP: anti-U1-RNP antibody; aSS-A/Ro: anti-SS-A antibody.

has not yet been reported, we determined, using our own healthy cohorts, that *HLA-DRB5*01:05* is associated with SSc-related ILD, with odds ratios ranging from 8.07 to 17.39 (Table 4). Our multivariate analysis also identified *HLA-DRB5*01:02* and anti-Topo-I as risk factors for SSc-related ILD (supplementary data Table 2, available as supplementary data at *Rheumatology* Online). Although the *HLA-DRB5* locus is almost exclusively carried by *HLA-DRB1*15* and *HLA-DRB1*16* haplotypes, neither of these alleles were related to ILD (supplementary data Table 1, available as supplementary data at *Rheumatology* Online). The lack of correlation between *HLA-DRB5*01:05* and any of the investigated autoantibodies suggests the existence of unknown or unstudied autoantibodies strongly related to ILD in SSc. Further investigation is necessary to identify the lung-related self antigen(s) recognized by HLA haplotypes, including this DRB5 allele. Of note, the *HLA-DRB5*01:05* allele is not more frequent in SSc patients without ILD relative to healthy controls. Although the possibility remains that another molecule in linkage disequilibrium with this *HLA-DRB5* allele is responsible for the development of ILD in SSc patients, the increase in the *HLA-DRB5* transcripts in SSc-ILD patients reveals that *HLA-DRB5* itself is a risk for this visceral complication.

In conclusion, we determined in two Japanese cohorts that *HLA-DRB5* gene carriers were more frequent in SSc patients with ILD than in those without ILD or in healthy controls. In particular, the *HLA-DRB5*01:05* allele was more frequent in SSc patients with ILD than in those without, with stronger statistical significance. *HLA-DRB5*01:05* is a novel candidate for a risk factor for developing ILD in patients with SSc.

**Rheumatology key messages**

- *HLA-DRB5* transcripts were up-regulated in peripheral blood of SSc patients with ILD.
- *HLA-DRB5* carriers were more prevalent in SSc patients with ILD than in those without.
- The *HLA-DRB5*01:05* allele is a risk for the development of ILD in patients with SSc.

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## Supplementary data

Supplementary data are available at *Rheumatology* Online.

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RESEARCH ARTICLE Open Access

# A novel autoantibody against fibronectin leucine-rich transmembrane protein 2 expressed on the endothelial cell surface identified by retroviral vector system in systemic lupus erythematosus

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## Abstract

**Introduction:** Anti-endothelial cell antibodies (AECAs) are thought to be critical for vasculitides in collagen diseases, but most were directed against molecules localized within the cell and not expressed on the cell surface. To clarify the pathogenic roles of AECAs, we constructed a retroviral vector system for identification of autoantigens expressed on the endothelial cell surface.

**Methods:** AECA activity in sera from patients with collagen diseases was measured with flow cytometry by using human umbilical vein endothelial cells (HUVECs). A cDNA library of HUVECs was retrovirally transfected into a rat myeloma cell line, from which AECA-positive clones were sorted with flow cytometry. cDNA of the cells was analyzed to identify an autoantigen, and then the clinical characteristics and the functional significance of the autoantibody were evaluated.

**Results:** Two distinct AECA-positive clones were isolated by using serum immunoglobulin G (IgG) from a patient with systemic lupus erythematosus (SLE). Both clones were identical to cDNA of fibronectin leucine-rich transmembrane protein 2 (*FLRT2*). HUVECs expressed FLRT2 and the prototype AECA IgG bound specifically to *FLRT2*-transfected cells. Anti-FLRT2 antibody activity accounted for 21.4% of AECAs in SLE. Furthermore, anti-FLRT2 antibody induced complement-dependent cytotoxicity against FLRT2-expressing cells.

**Conclusions:** We identified the membrane protein FLRT2 as a novel autoantigen of AECAs in SLE patients by using the retroviral vector system. Anti-FLRT2 antibody has the potential to induce direct endothelial cell cytotoxicity in about 10% of SLE patients and could be a novel molecular target for intervention. Identification of such a cell-surface target for AECAs may reveal a comprehensive mechanism of vascular injury in collagen diseases.

## Introduction

Vascular endothelial cells (ECs) represent the boundary between blood and tissue, and contribute to the process of inflammation. Anti-endothelial cell antibodies (AECAs) were first described in 1971 and defined as autoantibodies that target antigens present on the EC membrane [1,2]. AECAs have been detected in a number of patients with collagen diseases, including systemic lupus erythematosus (SLE), and were shown to be correlated to disease activity [3,4]. SLE is one of the diseases in which AECAs are frequently detected, and they are considered to play a role in the pathogenesis, especially in lupus nephritis [3,4]. In addition, SLE patients have an increased risk of cardiovascular disease originating from SLE itself, and it has been reported that AECAs play roles in atherosclerotic events [5].

AECAs have the potential to induce vascular lesions directly because their targets are expressed on ECs, which are always in contact with these circulating antibodies. AECAs are considered to play roles in the

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development of pathologic lesions by EC cytotoxicity (complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC)), activation of EC (proinflammatory cytokine secretion and expression of adhesion molecules), induction of coagulation, and induction of apoptosis [6-9].

Although new biologic drugs have been applied to the treatment of SLE, currently available therapies often introduce the additional risk of immunosuppression [10]. Bloom *et al.* [11] proposed a model for customized and specific therapeutic approaches against a highly pathogenic subset of lupus antibodies by using small molecules that neutralize them. AECAs may be good targets for such interventions, and identification of cell-surface targets of AECAs is required.

Target antigens of AECAs had been investigated intensively, but they are heterogeneous and classified into the following three groups: membrane component, ligand-receptor complex, and molecule adhering to the plasma membrane [12]. The cellular localization of the target antigen is considered to be a critical factor in the pathogenesis of autoantibodies [13], and it is generally accepted that autoantibodies against integral membrane proteins are usually pathogenic [14]. Although AECAs must be directed against the cell surface, most of the molecules reported to date as targets for AECAs are intracellular proteins [2,4,6,15]. Several groups have recently identified targets of AECAs by proteomics analysis [16,17]. However, extraction of some membrane proteins is difficult in proteomics analysis, and this may be one of the reasons that such proteins were not identified as AECA targets [2].

We constructed a retroviral vector system [18] to identify autoantigens expressed on the EC surface by using flow cytometry and identified the membrane protein fibronectin leucine-rich transmembrane protein 2 (FLRT2) as a novel autoantigen of AECAs in patients with SLE.

## Materials and methods

### Sources of human sera

Two hundred thirty-three patients with collagen diseases (196 female and 37 male patients) were enrolled in the study. The mean age was 42.5 years, with a range of 18 to 72 years. The patients comprised 95 with SLE and 138 with other collagen diseases. All of the patients were diagnosed according to the respective classification criteria [19-32]. Thirty-five age- and sex-matched healthy donors were enrolled as a control group. Sera were collected and stored at -20°C until use. All subjects gave written consent after the purpose and potential risks involved in the study were explained. The study protocol complied with the principles of the Declaration of Helsinki and was approved by the Ethical Committee of Tohoku University Graduate School of Medicine.

### Cell culture

Human umbilical vein endothelial cells (HUVECs), human aortic endothelial cells (HAECs), human lung microvascular endothelial cells (HMVEC-Ls), and EGM-2 medium were purchased from Lonza (Basel, Switzerland). Human renal glomerular endothelial cells (HRGECs) and endothelial cell medium were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). The cells were grown in 5% $CO_2$ at 37°C on polystyrene flasks (BD Biosciences, Bedford, MA, USA). These ECs were used at sooner than the fifth passage. HEK293T cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA), Plat-E and Plat-GP packaging cells were purchased from Cell Biolabs (San Diego, CA, USA) and cultured in Dulbecco modified Eagle medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT, USA). Rat myeloma cells, YB2/0, were purchased from ATCC and cultured in RPMI1640 medium (Sigma) containing 10% FBS.

### IgG purification

IgG fractions were purified from sera by using HiTRAP Protein G HP columns (Amersham Biosciences, Roosendaal, The Netherlands). The concentration of purified IgG was determined by measuring the OD at 280 nm ($OD_{280}$). Purified IgG was stored at -20°C until use.

### Flow cytometry

Binding activities of antibodies to the surface of ECs and FLRT2 molecules were measured by using FACSCalibur and FACSCanto II (Becton Dickinson, Franklin Lakes, NJ, USA) [17], and the data were analyzed with FlowJo Software (Tree Star, Ashland, OR, USA). In brief, attached cells were dissociated from plates by using Cell Dissociation Solution (Sigma) and washed with phosphate-buffered saline (PBS). Aliquots of $1 \times 10^5$ cells/tube were incubated in blocking buffer (PBS containing 1% bovine serum albumin and 50 mg/ml goat gamma globulin fraction (Sigma)) with primary antibodies at 4°C for 30 minutes. After washing, cells were incubated with secondary antibodies and 7-amino-actinomycin D (7-AAD) (BD Biosciences) at 4°C for 30 minutes and analyzed with flow cytometry.

Primary antibodies included 1:10 diluted human serum, 0.5 mg/ml of purified human IgG, and 10 μg/ml goat anti-human FLRT1/FLRT2/FLRT3 antibody (R&D Systems, Minneapolis, MN, USA). Secondary antibodies included 1:50 diluted fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-conjugated goat anti-human IgG (Abcam, Cambridge, UK), PE-conjugated donkey anti-goat IgG (Abcam), PE-conjugated mouse anti-human IgG1/IgG2/IgG3/IgG4 antibody (Beckman Coulter, Fullerton, CA, USA), and DyLight 650-conjugated anti human IgM antibody (Abcam). For staining of the intracellular FLRT2

domain, IntraStain (Dako, Glostrup, Denmark) and anti-human FLRT2 antibody (K-20) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used.

For measurement of AECA activity, the relative mean fluorescence intensity (MFI) ratio was calculated as follows: (sample MFI - control MFI)/control MFI × 100 [33]. Relative MFI ratio of mean + 3 standard deviations (SD) among the control group was defined as the cutoff value for AECAs. For measurement of anti-FLRT2 activity against the cell-surface domain, the relative MFI ratio was calculated as follows: (MFI against FLRT2-expressing cells - MFI against non-FLRT2-expressing cells)/MFI against non-FLRT2-expressing cells × 100. In each set of experiments, relative MFI ratios of titrated reference serum with high anti-FLRT2 activity were calculated, and a standard curve was generated. The relative MFI ratio was converted to arbitrary units (AUs) according to the standard curve. AU of mean + 3 SD in the control group was defined as the cutoff value for the anti-FLRT2 antibody. Recombinant human FLRT2 (R&D Systems) was added at the indicated dose in inhibition tests. The percentage inhibition was calculated as follows: % inhibition = (AECA titer of sample serum - AECA titer of sample serum with inhibitor)/AECA titer of sample serum × 100.

### HUVEC cDNA library

Total RNA was generated from HUVECs by using an RNeasy Mini Kit (Qiagen, Hilden, Germany), and poly(A) + RNA was purified with an mRNA Purification Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Double-stranded cDNA was synthesized by using a cDNA library construction kit (Takara Bio, Shiga, Japan). DNA fragments > 1,000 bp in length were ligated into the pMX vector (kindly donated by Toshio Kitamura, University of Tokyo, Tokyo, Japan).

### Screening of cDNA library

The HUVEC cDNA library in pMX was retrovirally transfected into the YB2/0 rat myeloma cell line [34]. Aliquots of $1 \times 10^7$ YB2/0 cells expressing the HUVEC cDNA library were incubated with 0.5 mg/ml of IgG with high AECA activity at 4°C for 30 minutes. After washing, cells were incubated with FITC-conjugated goat anti-human IgG and 7-AAD at 4°C for 30 minutes. The cells showing a high level of FITC fluorescence signal were sorted with FACS Vantage (Becton Dickinson). Sorted cells were kept in culture until the cell number increased sufficiently for the next round of sorting. Subcloning of cells bound to IgG with AECA activity was performed by the limiting dilution method.

Genomic DNAs of clones were purified by using the Wizard SV Genomic DNA Purification system (Promega Corporation, Madison, WI, USA). DNA fragments from the HUVEC cDNA library were amplified by polymerase chain reaction (PCR) by using TaKaRa LA Taq (Takara Bio) with primers corresponding to the 5' and 3' ends of the multiple cloning site of pMX (5'-GGTGGACCATCCTCTAGACTG, 3'-CCTTTTTCTGGAGACTAAAT, respectively). The PCR products were cloned into the pCR-TOPO vector (Invitrogen), and DNA sequences were analyzed with the BLAST program.

### Expression of FLRT2 in HEK293T cells

The full-length FLRT2 fragment was amplified by PCR from genomic DNA of FLRT2-expressing YB2/0 clone sorted as described earlier, by using Phusion High-Fidelity DNA Polymerase (Finnzymes, Keilaranta, Espoo, Finland). Primer sequences were as follows: 5'-CCCACCACATTGTATTTTATTTCC, 3'-CTTGATAACGCTGGGCCTCT. The FLRT2 fragment was inserted into the pMX-IRES-GFP vector (Cell Biolabs). An FLRT2 expression vector with deletion of the unique region was made by using an In-Fusion HD Cloning Kit (Clontech Laboratories, Madison, WI, USA) with two PCR segments constructed to omit the unique region (363 to 419 amino acids) and inserted into the pMX-IRES-GFP vector. pMX-FLRT2-IRES-GFP was transfected directly into HEK293T cells with FuGENE HD (Roche Diagnostics, Basel, Switzerland) or retrovirally transfected into HEK293T cells. Full-length FLRT1 and FLRT3 fragments were amplified as described earlier and inserted into the pMX-IRES-GFP vector.

### Western blotting

Cells were lysed in RIPA buffer (Cell Signaling Technology, Danvers, MA, USA). The lysate was mixed with 5 × sodium dodecyl sulfate (SDS) sample buffer and separated by electrophoresis on an 8% polyacrylamide gel. The proteins were then transferred onto Immobilon Transfer Membranes (Millipore, Billerica, MA, USA). The membranes were treated with 0.1 μg/ml of goat anti-FLRT2 antibody and IRDye680-conjugated donkey anti-goat IgG (LI-COR Biosciences, Lincoln, NE, USA), and fluorescence intensity was determined with the Odyssey Infrared Imaging System (LI-COR).

### CDC

CDC was assessed by the tetrazolium salt reduction method by using WST-1 (Roche Diagnostics) [35-37]. In brief, cells were seeded in 96-well culture plates at a concentration of $4 \times 10^4$ cells per well and cultured overnight. Cells were incubated with 100 μl of diluted IgG for 30 minutes followed by addition of 50 μl of rabbit complement (Cedarlane Laboratories, Burlington, ON, Canada) at the indicated concentrations for 2 hours at 37°C. Then 15 μl of WST-1 was added, and cells were incubated for an additional 4 hours. Absorbance at 450 nm ($A_{450}$) was measured and expressed as relative fluorescence units (RFUs), reflecting the number of viable cells. Triton X-100 (1%)

and heat-inactivated complement were added to the wells to measure background or maximal absorbance of WST-1, respectively. Recombinant FLRT2 was added in the inhibition tests. The percentage cytotoxicity for each sample was calculated by using the formula:

$$\% \text{ cytotoxicity} = (\text{maximal RFU} - \text{sample RFU})/(\text{maximal RFU} - \text{background RFU}) \times 100.$$

### ADCC

ADCC was determined by using the LDH Cytotoxicity Detection Kit (Takara Bio) and the manufacturer's protocol [36].

The percentage cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = (\text{experimental} - \text{effector spontaneous} - \text{target spontaneous})/(\text{target maximum} - \text{target spontaneous}) \times 100.$$

### Detection of adhesion molecule expression

HUVECs were cultured overnight in 96-well culture plates and incubated with IgG (640 μg/ml) for 6 hours at 37°C. Harvested cells were stained with PE-conjugated anti-CD62E antibody (BioLegend, San Diego, CA, USA), allophycocyanin (APC)-conjugated anti-CD106 antibody (BioLegend), and Pacific blue-conjugated anti-CD54 antibody (BioLegend), and were analyzed with flow cytometry.

### Detection of EC apoptosis

HUVECs were seeded in 48-well culture plates and incubated with test IgG (640 μg/ml) for 24 hours, and apoptosis in the harvested cells was measured with annexin V and 7-AAD (Apoptosis Detection Kit; BD Biosciences) with flow cytometry. Annexin V-positive/7-AAD-negative cells were measured as apoptotic cells.

### Statistical analysis

Data were analyzed by using the two-tailed Student *t* test or Mann-Whitney *U* test for continuous variables. Pairwise comparisons were assessed by using the Wilcoxon signed-rank test. Spearman rank correlation was used to explore the associations between anti-FLRT2 titer and clinical parameters. All analyses were performed by using Prism software (GraphPad Software, La Jolla, CA, USA). In all analyses, $P < 0.05$ was taken to indicate statistical significance.

## Results

### Detection of AECA activity with flow cytometry

We first examined AECA activity in the sera from patients with collagen diseases by measuring binding activity of IgG to nonpermeabilized 7-AAD-negative HUVECs by using flow cytometry. The prevalence of AECAs was significantly higher in patients with SLE (50.5%) and other collagen diseases compared with normal controls (2.9%) (Figure 1). As these data indicated the presence of autoantigens on the EC surface, we constructed a retroviral vector system to identify cell-surface target molecules of AECAs with flow cytometry.

### Sorting of cells expressing cell-surface autoantigens with retroviral vector system

Among sera with AECA activity, one sample (E10-19) from an SLE patient with active lupus nephritis (WHO IV) showed strong AECA activity (Figure 2A). We selected this serum sample as the prototype of AECAs for subsequent cell sorting. Purified IgG from E10-19 serum also showed strong binding to the surface of HUVECs, and IgG from the same patient collected after the treatment with 1 mg/kg prednisolone and intravenous cyclophosphamide showed remarkably reduced AECA activity (Figure 2A).

The YB2/0 cell line expressing HUVEC cDNA was generated by stable transfection of the HUVEC cDNA library with the retroviral vector system. After staining of this cell line with E10-19 IgG and FITC-conjugated secondary antibody, cells with strong FITC signals were sorted with flow cytometry. After cell expansion, we repeated one more round of cell sorting to concentrate E10-19 IgG-binding cells (Figure 2B). After the second sorting, cells bound to E10-19 IgG were markedly increased, and several cell clones were established from the E10-19 IgG-binding cell population by the limiting dilution method. Two distinct clones with different E10-19 IgG-binding activities and gene profiles of transfected HUVEC cDNA were established, C9 and C18 (Figure 2C and 2D).

### Identification of FLRT2 as a novel cell-surface autoantigen

After PCR amplification and cloning of HUVEC cDNA inserted into the genomic DNA of C9 and C18, DNA sequencing was performed followed by BLAST analysis. PCR bands of around 3,000 bp in C9 and C18 (Figure 2D, black box) were found to be an identical gene, that is, fibronectin leucine-rich transmembrane protein 2 (*FLRT2*) cDNA (GenBank accession number NM_013231.4). Real-time quantitative PCR and microarray analysis of YB2/0, C9, and C18 also supported the conclusion that only the *FLRT2* mRNA was overexpressed in both C9 and C18 (data not shown). Flow cytometry and Western blotting showed that FLRT2 protein was expressed on the cell surfaces of C9 and C18 (Figure 3A). Next, we generated an expression vector of FLRT2, which was transfected into HEK293T cells. E10-19 IgG showed significant binding activity to 7-AAD-negative FLRT2-expressing HEK293T cells (Figure 3B), indicating that E10-19 IgG has high anti-FLRT2 activity. Thus, the membrane protein FLRT2 was identified as a novel autoantigen.

Flow cytometry and Western blotting indicated that HUVECs and other ECs also expressed significant levels

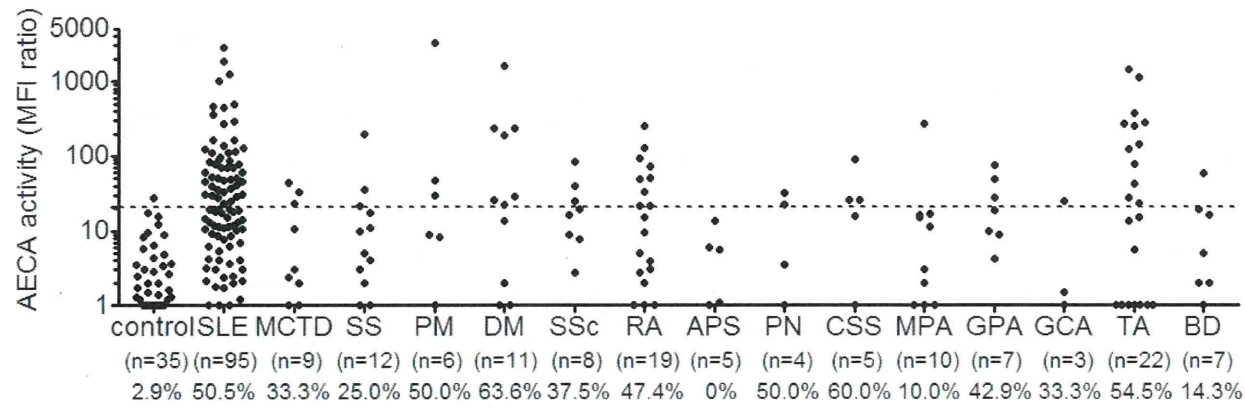


**Figure 1 Distribution of antiendothelial cell antibodies (AECAs)**. The distribution of AECAs in collagen diseases was measured with flow cytometry. Dots represent the data for individual subjects. The broken horizontal line indicates the cutoff value for high titers of AECAs (mean + 3 SD). Collagen diseases included systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD), Sjögren syndrome (SS), polymyositis (PM), dermatomyositis (DM), systemic sclerosis (SSc), rheumatoid arthritis (RA), antiphospholipid syndrome (APS), polyarteritis nodosa (PN), Churg-Strauss syndrome (CSS), microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA), giant cell arteritis (GCA), Takayasu arteritis (TA), and Behçet disease (BD).

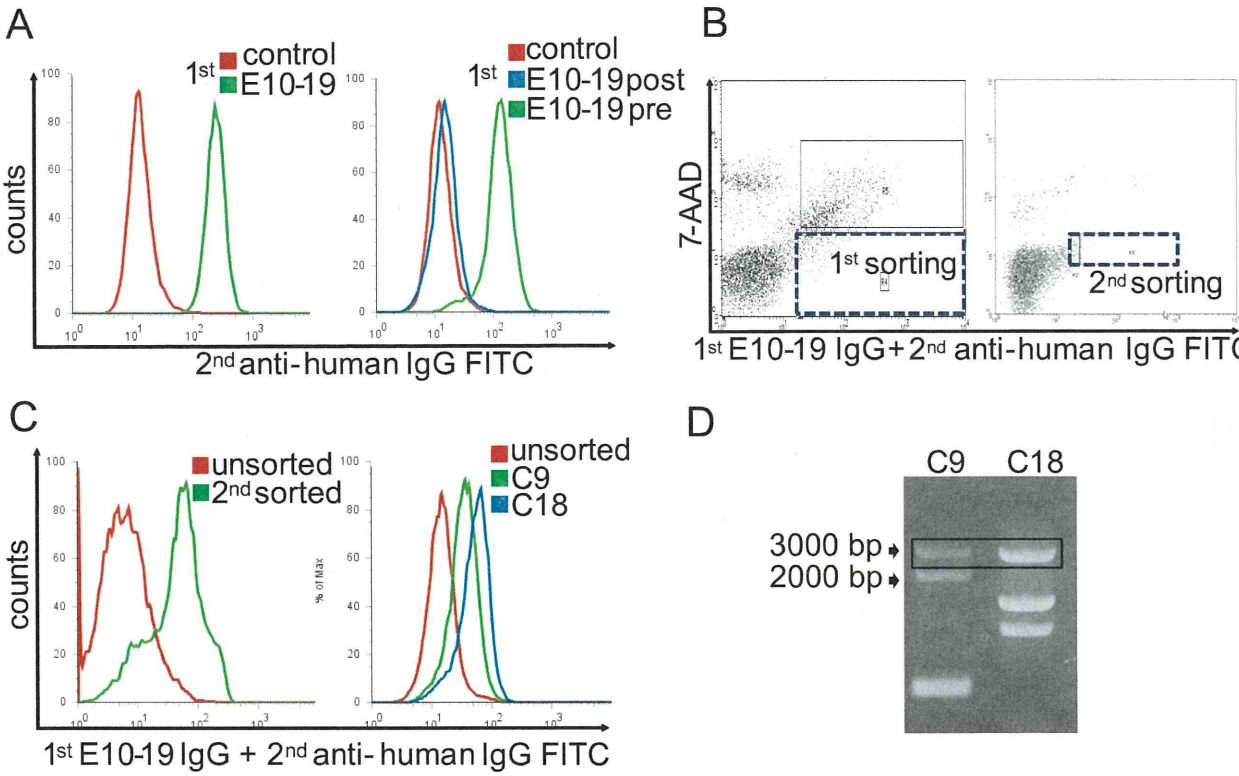


**Figure 2 Subcloning of autoantigen-expressing cells by using IgG from a patient with lupus nephritis**. **(A)** Nonpermeabilized HUVECs were stained with 1:10 diluted sera of control or E10-19 from a lupus nephritis patient (left), and 0.5 mg/ml of IgG of control or E10-19 collected before (pre) or after (post) the treatments (right) followed by secondary antibody and analyzed with flow cytometry. **(B)** HUVEC cDNA-expressing cells were stained with 0.5 mg/ml of E10-19 IgG followed by secondary antibody, and cells in the positive fraction were sorted (black dotted box). Left indicates first sorting, and right indicates second sorting. **(C)** Unsorted and second-sorted cells (left), and unsorted and two clones from second-sorted cells, C9 and C18, respectively (right), were stained with 0.5 mg/ml of E10-19 IgG followed by secondary antibody, and analyzed with flow cytometry. **(D)** HUVEC cDNA fragments inserted into the genomic DNA of C9 and C18 were amplified, and PCR products were electrophoresed on an 0.8% agarose gel.

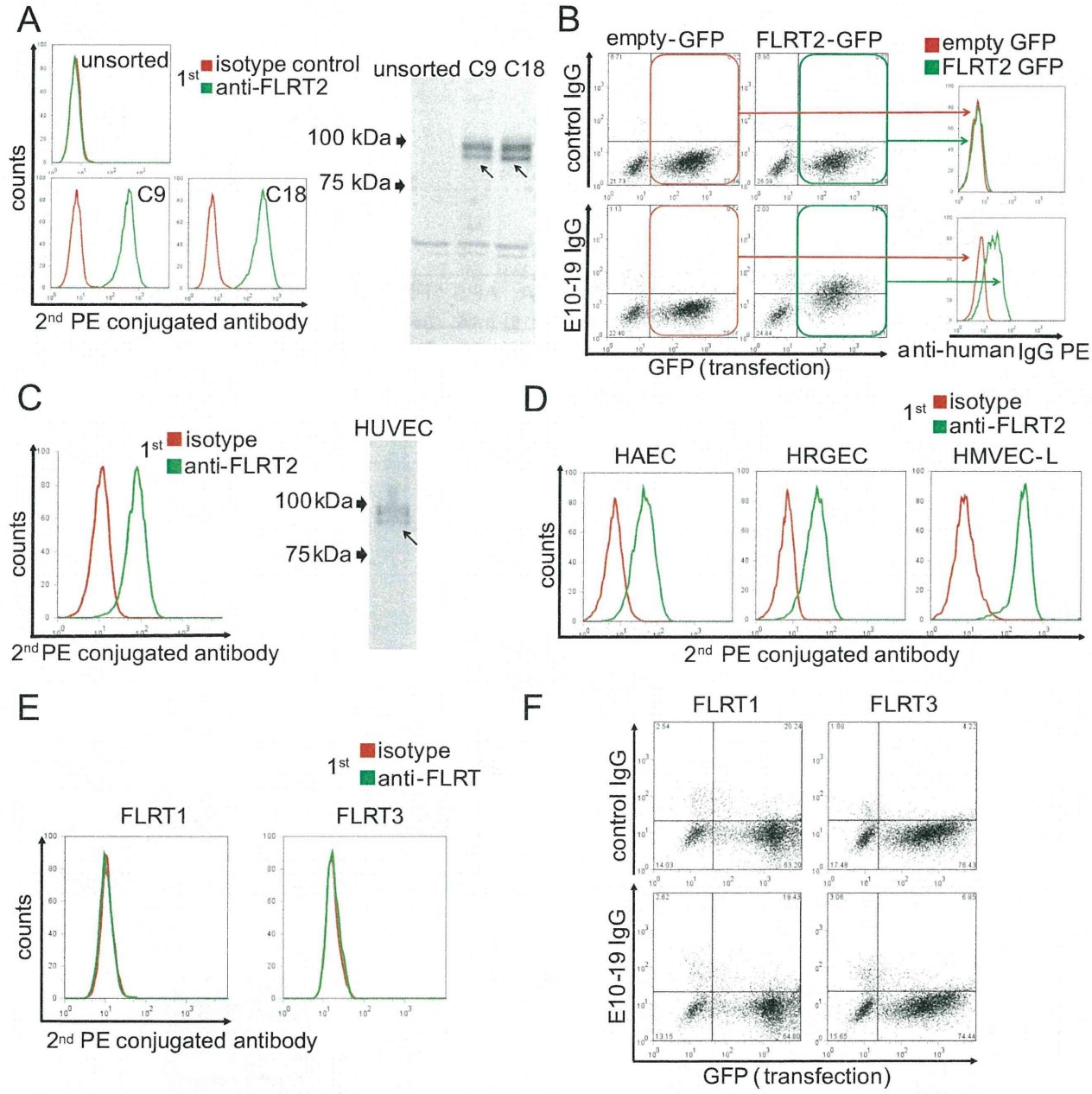


**Figure 3 Identification of FLRT2 as a novel autoantigen of AECAs. (A)** Unsorted, C9, and C18 were stained with isotype control or anti-FLRT2 antibody, followed by secondary antibody, and analyzed with flow cytometry (left). Western blotting of proteins from unsorted, C9, and C18 was performed, and they were stained with anti-FLRT2 antibody followed by secondary antibody (right). Arrows indicate the bands of FLRT2. Both of the two bands are FLRT2 because some of FLRT2 proteins were glycosylated. **(B)** Expression vector, empty-IRES-GFP, or FLRT2-IRES-GFP was transfected into HEK293T cells, and these cells were stained with 0.5 mg/ml of control IgG or E10-19 IgG, followed by secondary antibody, and analyzed with flow cytometry. Binding activities of IgG to cell-surface FLRT2 were analyzed in histograms (right) by gating for the GFP-positive transfected population (left). **(C)** HUVECs were stained with isotype control or anti-FLRT2 antibody followed by secondary antibody, and analyzed with flow cytometry (left). Western blotting of proteins from HUVECs was performed, and they were stained with anti-FLRT2 antibody followed by secondary antibody (right). The arrows indicate the bands of FLRT2. **(D)** HAECs, HRGECs, and HMVEC-Ls were stained with isotype control or anti-FLRT2 antibody followed by secondary antibody, and analyzed with flow cytometry. **(E)** HUVECs were stained with isotype control, anti-FLRT1 antibody, or anti-FLRT3 antibody followed by secondary antibody, and analyzed with flow cytometry. **(F)** Expression vector, FLRT1-IRES-GFP (left), or FLRT3-IRES-GFP (right) was transfected into HEK293T cells, and these cells were stained with 0.5 mg/ml of control IgG or E10-19 IgG, followed by secondary antibody, and analyzed with flow cytometry.

of FLRT2 on their cell surfaces (Figure 3C, D). FLRT2 is a member of the FLRT family, which includes FLRT1, FLRT2, and FLRT3 [38]. We examined whether other FLRTs were expressed on HUVECs with flow cytometry. Neither FLRT1 nor FLRT3 was expressed on the surface of these ECs, and E10-19 IgG showed no binding activity to either FLRT1 or FLRT3 (Figure 3E, F). These data indicated that among the FLRT family, FLRT2 was the only target molecule of AECAs.

### Inhibition test and epitope mapping

We conducted inhibition tests to determine whether the AECA activities of anti-FLRT2-positive SLE patients were due to anti-FLRT2 activity. Incubation with soluble recombinant FLRT2 inhibited the binding of patient IgG to HUVECs (Figure 4A). We further investigated the epitope of anti-FLRT2 antibody. FLRT2 contains extracellular leucine-rich repeats, unique region, fibronectin type III domain, and a cytoplasmic tail. As mentioned earlier, FLRT2 was the only member of the FLRT family that was bound by SLE IgG, so we hypothesized that the unique region of FLRT2 may be the major epitope for anti-FLRT2 antibody. To investigate this hypothesis, an expression vector of FLRT2 lacking the unique region (FLRT2Δur) was generated. As shown in Figure 4B, the binding activity of the anti-FLRT2 antibody was significantly reduced when FLRT2 lacked its unique region ($P$ = 0.008) compared with the equal binding activity of anti-FLRT2 antibody to the intracellular domain. These observations indicated that the major epitope was localized within the unique region of FLRT2.

### Distribution of patients with anti-FLRT2 activity

Anti-FLRT2 activities were detected in nine (10.2%) of 88 patients with SLE and one (6.7%) of 15 patients with granulomatosis with polyangiitis (Wegener's). Healthy controls and other patients with collagen diseases, including diseases that showed a high prevalence of AECA activity, did not show anti-FLRT2 activity (Figure 5A). Strong anti-FLRT2 activities were detected in only SLE patients, indicating that anti-FLRT2 antibody is specific to SLE patients. Among 48 SLE patients with AECA positivity (Figure 1), 42 were examined for anti-FLRT2 activity, and nine patients (21.4%) were positive.

Among SLE patients with anti-FLRT2 positivity, anti-FLRT2 activity was significantly correlated with low levels of complement C3, C4, and CH50 (Figure 5B). No interrelations were found between anti-FLRT2 activity and the SLE disease activity index (SLEDAI), anti-dsDNA antibody titer, or serum amyloid A (SAA) level (Figure 5B).

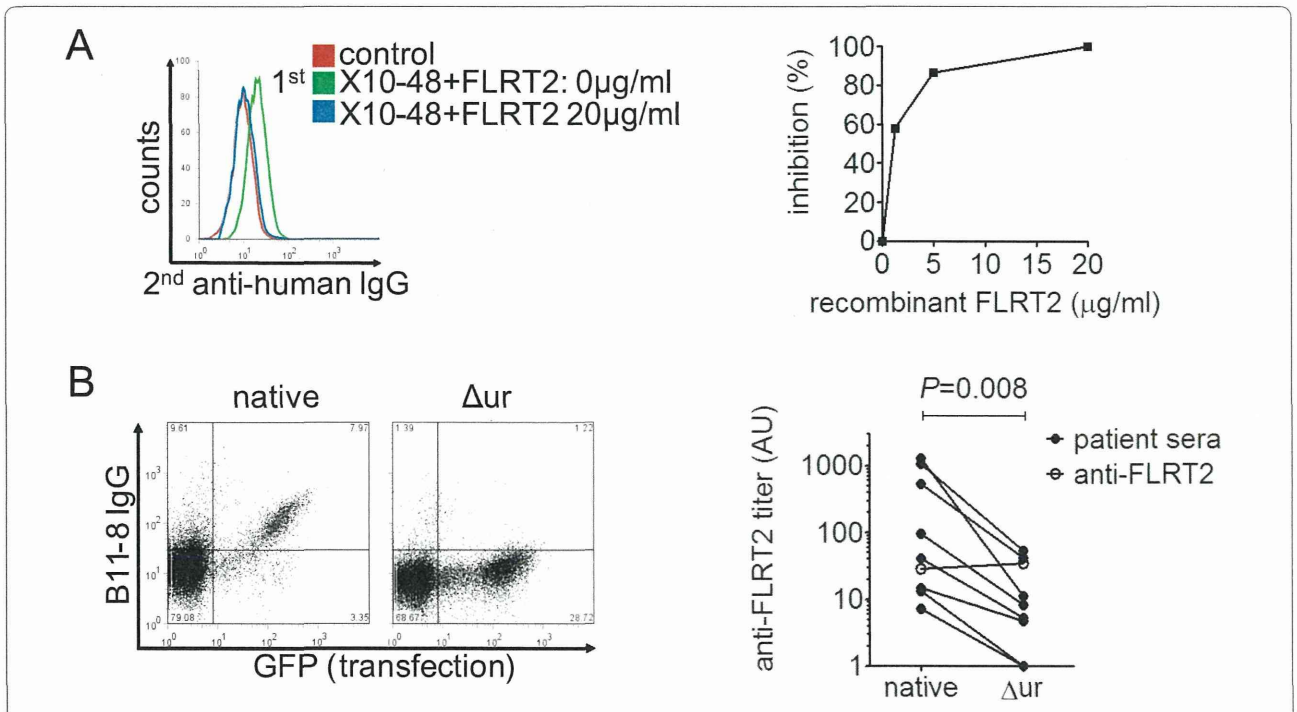


**Figure 4 Inhibition test and epitope mapping**. **(A)** Inhibition tests of binding activities to HUVECs were performed by using IgG from healthy donor (control) and anti-FLRT2 sera (X10-48) with soluble FLRT2 at the indicated concentrations. **(B)** Changes in binding activity to FLRT2 lacking the unique region (Δur) compared with native FLRT2 were analyzed by using anti-FLRT2 sera. Representative dot plot (left) and a summary of changes in each patient (right) are shown. Open circles show the binding activity to the intracellular FLRT2 domain.