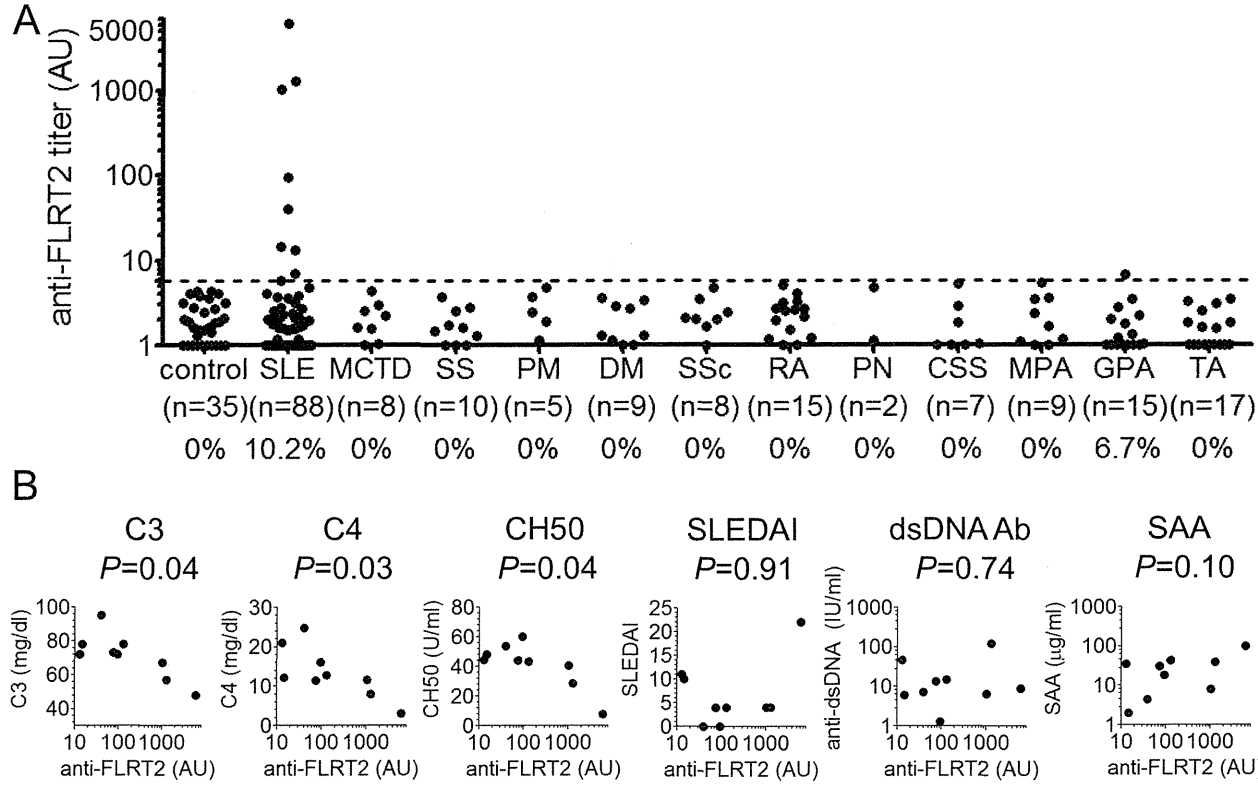

Figure 5 Distribution of patients with anti-FLRT2 activity. (A) The distribution of anti-FLRT2 activity in collagen diseases was measured with flow cytometry. Dots represent the data for individual subjects. The broken horizontal line indicates the cutoff value for high anti-FLRT2 activity (mean + 3SD). 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), polyarteritis nodosa (PN), Churg-Strauss syndrome (CSS), microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA), and Takayasu arteritis (TA). **(B)** Correlations of clinical parameters with anti-FLRT2 activity among anti-FLRT2-positive SLE patients are shown. CH50, 50% hemolytic complement activity; SLEDAI, SLE disease activity index; dsDNA Ab, anti-double-stranded DNA antibody; SAA, serum amyloid A.

Induction of endothelial cell killing by CDC

We next assessed the functional significance of anti-FLRT2 antibody by using IgG from the sera of two SLE patients with high FLRT2 activity (B11-8 and X10-48). IgG with anti-FLRT2 activity showed significant CDC activity against HUVECs compared with IgG from normal controls (Figure 6A). This CDC activity was inhibited by incubation with soluble recombinant FLRT2, and increased with a higher concentration of IgG (Figure 6B, C). Strong CDC activity was induced against FLRT2-expressing HEK293T cells, but not against mock-transfected HEK293T cells (Figure 6D). These observations confirmed the ability of the anti-FLRT2 antibody to induce CDC activity by binding to cell-surface FLRT2.

We also analyzed the IgG subclasses of anti-FLRT2 antibody with flow cytometry. In all anti-FLRT2 active IgG subclasses, IgG1 and IgG2 activities were strong, and IgG3 was weak. The presence of IgG4 varied between patients (Figure 6E). Compared with IgG, weak IgM activity was detected (Figure 6F). None of these IgGs showed ADCC (Figure 6G).

Other pathogenic roles as AECAs

We examined further potentials for pathogenicity against EC activation and induction of apoptosis. The levels of expression of adhesion molecules (intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin) on HUVECs were not increased by incubation with IgG purified from B11-8 and X10-48 compared with control IgG (Figure 7A). Incubation of HUVECs with anti-FLRT2-positive IgG did not induce apoptosis (Figure 7B).

Discussion

Although the existence of AECAs in patients with SLE and other collagen diseases has been reported, its pathogenic significance remains unknown. The localization of the AECA target molecule on the cell surface should be

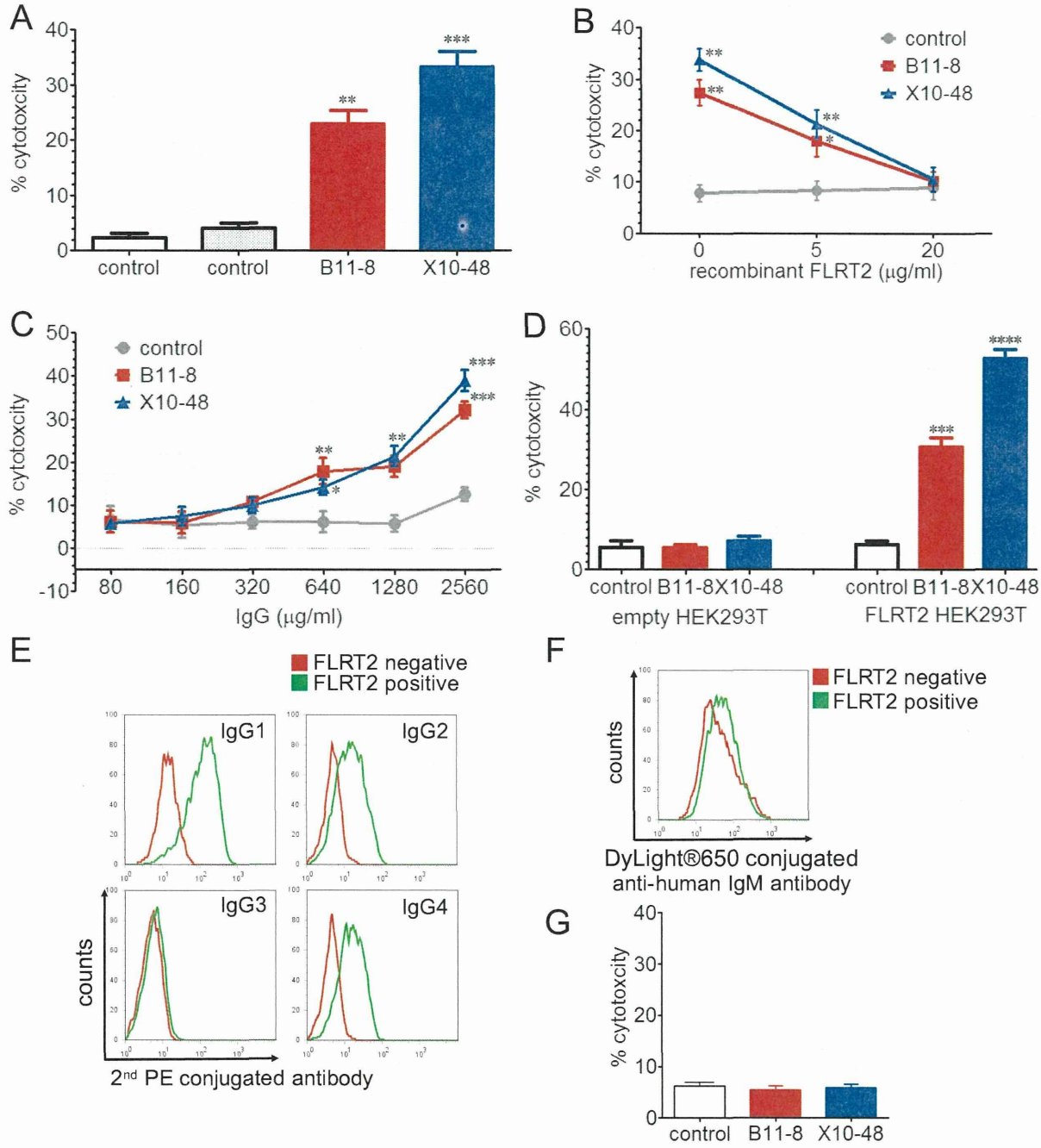

Figure 6 Complement-dependent cytotoxicity (CDC) of anti-FLRT2 antibody. CDC activities using two healthy control IgG and two anti-FLRT2 positive IgG, B11-8 and X10-48, at a concentration of 1.28 mg/ml with 1:3 diluted complement **(A)**, 1.28 mg/ml of IgG, and 1:3 diluted complement with recombinant FLRT2 at the indicated concentrations **(B)**, and various IgG concentrations with 1:6 diluted complement **(C)** against HUVECs were measured with the WST-1 assay. **(D)** CDC activities against mock transfected HEK293T cells (empty, left) and FLRT2-expressing HEK293T cells (FLRT2, right) by using 1.28 mg/ml of IgG and 1:3 diluted complement were measured with the WST-1 assay. HEK293T cells negative or positive for FLRT2 expression were stained with anti-FLRT2 antibody followed by secondary antibody against human IgG1, IgG2, IgG3, IgG4 **(E)**, and IgM **(F)**, and analyzed with flow cytometry. **(G)** ADCC activities using control IgG, B11-8, and X10-48, at a concentration of 1.28 mg/ml with an E:T ratio of 25:1 were determined with the lactate dehydrogenase detection method. Error bars indicate SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

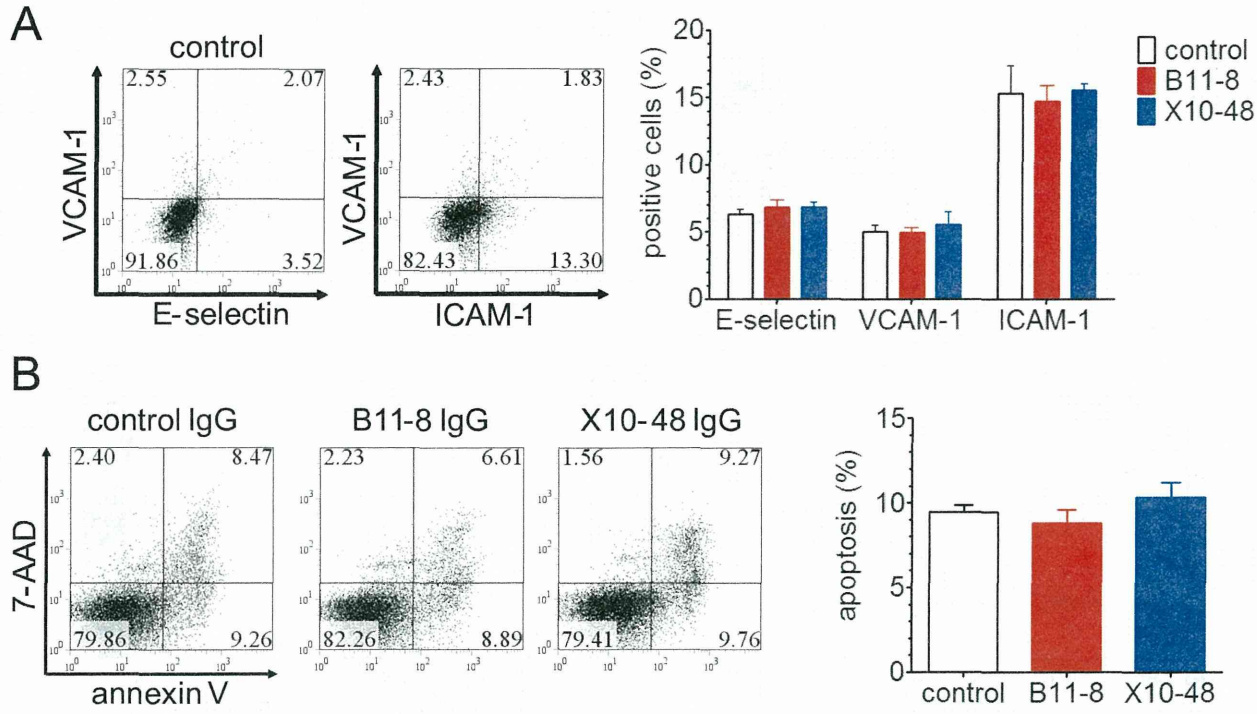

Figure 7 Activation of HUVECs and induction of apoptosis. (A) Expression of E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) was analyzed with flow cytometry against HUVECs treated with 640 μg/ml of control and two anti-FLRT2-positive IgGs, B11-8 and X10-48. Representative graphs (left) and summarized graph (right) are shown. **(B)** HUVECs treated with control IgG and two anti-FLRT2-positive IgGs, B11-8 and X10-48, for 24 hours were stained with annexin V and 7-AAD and analyzed with flow cytometry. (Annexin V positive/7-AAD negative) cells were measured as apoptotic cells. Representative graphs (left) and summarized graph (right) are shown. Error bars indicate SD.

an important factor for its pathogenicity *in vivo*, in terms of accessibility of the target molecule to AECAs.

Our strategy to identify AECA target molecules is to use a retroviral vector system and flow cytometry. As the localization of cellular molecules depends on their structures, only cell-surface molecules are expressed on the surface of YB2/0 cells transfected with the HUVEC cDNA library. AECAs can bind only to cell-surface molecules in flow cytometry. Therefore, sorting of IgG-binding cells can concentrate and isolate cells expressing autoantigens (target molecules for AECAs) on the cell surface. Although this system may present difficulties in sorting cells at very low frequency, we isolated and cloned autoantigen-expressing cells by repeated sorting, and this system was shown to be useful to identify cell-surface autoantigens. Whereas some cell-surface molecules were identified with this system previously [39], this is the first report of autoantigen identification.

With purified IgG from one SLE patient with high AECA activity (E10-19), two distinct clones were isolated and established, both of which were shown to have an identical gene, *FLRT2*. As we confirmed that E10-19 IgG bound specifically to cell-surface FLRT2 and FLRT2 was expressed on the cell surface of ECs, we concluded that FLRT2 is a novel cell-surface autoantigen as a target molecule for AECAs in SLE patients.

Analysis of anti-FLRT2 activity among patients with various collagen diseases indicated that anti-FLRT2 antibody was specifically detected in SLE, and it accounted for 21.4% of cell-surface target molecules of AECAs in SLE. AECA activity of IgG from SLE patients with anti-FLRT2 activity was significantly inhibited by soluble recombinant FLRT2, indicating that FLRT2 is the major target on ECs for AECAs in these patients. Although heat-shock protein 60 (Hsp60) has been described as the target antigen of AECAs in SLE and has a proapoptotic effect [40,41], Hsp60 was not detected on freshly isolated unstressed HUVECs [40,41]. The remaining 78.6% of SLE patients with AECA activity in the present study may have other as-yet-unidentified target antigens.

FLRT2 is transmembrane protein and was identified as a novel gene family in the screening for extracellular matrix proteins expressed in muscle [38]. Although FLRT2 was shown to be expressed in the pancreas, skeletal muscle, brain, and heart with Northern blotting [38], we confirmed the expression of FLRT2 on HUVECs and other ECs (HAECs, HRGECs, and HMVEC-Ls), and treatment with neither tumor necrosis factor-α (TNF-α)

nor lipopolysaccharide (LPS) induced the expression of FLRT2 (data not shown). E10-19 IgG did not bind to FLRT1 and FLRT3, and these two molecules were not expressed on ECs. Consistent with these findings, the major epitope for anti-FLRT2 antibody was localized in the unique region within the extracellular domain of FLRT2.

FLRT2 has been reported to modulate signaling, interact with fibroblast growth factor receptor, promote cell proliferation, participate in craniofacial development, and promote heart morphogenesis [42-46]. Although we hypothesized that anti-FLRT2 antibody may affect some cellular behavior and induce expression of adhesion molecules, cell proliferation, and apoptotic cell death without complement in ECs, we did not find these activities in the present study.

Among SLE patients with anti-FLRT2 activity, complement levels were correlated significantly with the anti-FLRT2 antibody titer. Moreover, anti-FLRT2 antibody induced cell damage in a complement-dependent manner, suggesting that it has pathogenic roles in immune-mediated vascular damage. CDC activity of AECAs was reported in patients with SLE, Takayasu arteritis, hemolytic-uremic syndrome, and Kawasaki disease [2,4,35,47,48]. Although ADCC activity was not proven in our study, similar observations of AECAs producing CDC but not ADCC were reported previously [35,48].

As demonstrated in this study, FLRT2 is widely distributed in various types of ECs. Therefore, it is possible that anti-FLRT2 antibody is linked to systemic vascular injury. These observations indicate that it is necessary to evaluate the contributions of anti-FLRT2 antibody to atherosclerotic lesions because chronic inflammation is atherogenic in SLE [49,50]. Administration of gammaglobulin was reported to reduce CDC of AECAs against ECs [35], and this may apply to anti-FLRT2 antibody-induced damage. Furthermore, incubation with soluble recombinant FLRT2 inhibited the AECA activity and CDC activity in patients with anti-FLRT2 positivity, which suggests that neutralizing anti-FLRT2 antibodies might be the specific therapeutic approach.

Conclusions

We identified the membrane protein FLRT2 as a novel autoantigen of AECAs in SLE patients. Our retroviral vector system is useful for identification of cell-surface autoantigens. In addition to further investigations of the biologic significance of anti-FLRT2 antibody and its therapeutic applications, other cell-surface autoantigens of AECAs should be determined to achieve a comprehensive understanding of AECA-mediated vascular injury and the development of more-specific intervention strategies.

Abbreviations

ADCC: antibody-dependent cell-mediated cytotoxicity; AECAs: anti-endothelial cell antibodies; APC: allophycocyanin; AUs: arbitrary units; CDC: complement-dependent cytotoxicity; DMEM: Dulbecco modified Eagle medium; ECs: endothelial cells; FBS: fetal bovine serum; FITC: fluorescein isothiocyanate; FLRT: fibronectin leucine-rich transmembrane protein; HAECs: human aortic endothelial cells; HMVEC-Ls: human lung microvascular endothelial cells; HRGECs: human renal glomerular endothelial cells; Hsp60: heat shock protein 60; HUVECs: human umbilical vein endothelial cells; ICAM-1: intercellular adhesion molecule 1; LPS: lipopolysaccharide; MFI: mean fluorescence intensity; PBS: phosphate-buffered saline; PCR: polymerase chain reaction; PE: phycoerythrin; RFUs: relative fluorescence units; SAA: serum amyloid A; SD: standard deviations; SDS: sodium dodecyl sulfate; SLE: systemic lupus erythematosus; SLEDAI: SLE disease activity index; TNF-α: tumor necrosis factor α; VCAM-1: vascular cell adhesion molecule 1; 7-AAD: 7-amino-actinomycin D.

Acknowledgements

We thank the staff of the Department of Hematology and Rheumatology, Tohoku University, for help and discussion, and Reika Saito for technical assistance. We also thank Prof. Masato Nose for critical reading of the manuscript. This work was supported in part by the Network Medicine Global-COE Program from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and Biomedical Research Core of Tohoku University Graduate School of Medicine.

Author details

[1]Department of Hematology and Rheumatology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-cho, Aoba-ku, Sendai, Miyagi 980-8574, Japan. [2]Department of Histopathology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-cho, Aoba-ku, Sendai, Miyagi 980-8574, Japan.

Authors' contributions

TS and HF carried out the molecular biologic studies, flow cytometry, clinical evaluation, and functional assays and drafted the manuscript. MO participated in the design of the study, performed the molecular biologic studies, and helped to draft the manuscript. KN, RW, YT, NT, and TI participated in its design and helped to draft the manuscript. HH conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 9 March 2012 Revised: 1 June 2012 Accepted: 2 July 2012
Published: 2 July 2012

References

1. Lindqvist KJ, Osterland CK: **Human antibodies to vascular endothelium.** *Clin Exp Immunol* 1971, **96**:753-760.
2. Guilpain P, Mouthon L: **Antiendothelial cells autoantibodies in vasculitis-associated systemic diseases.** *Clin Rev Allergy Immunol* 2008, **35**:59-65.
3. D'Cruz DP, Houssiau FA, Ramirez G, Baguley E, McCutcheon J, Vianna J, Haga HJ, Swana GT, Khamashta MA, Taylor JC, Davies DR, Hughes GR: **Antibodies to endothelial cells in systemic lupus erythematosus: a potential marker for nephritis and vasculitis.** *Clin Exp Immunol* 1991, **85**:254-261.
4. Renaudineau Y, Dugué C, Dueymes M, Youinou P: **Antiendothelial cell antibodies in systemic lupus erythematosus.** *Autoimmun Rev* 2002, **1**:365-372.
5. Soltesz P, Bereczki D, Szodoray P, Magyar MT, Der H, Csipo I, Hajas A, Paragh G, Szegedi G, Bodolay E: **Endothelial cell markers reflecting endothelial cell dysfunction in patients with mixed connective tissue disease.** *Arthritis Res Ther* 2010, **12**:R78.
6. Mihai C, Tervaert JW: **Anti-endothelial cell antibodies in systemic sclerosis.** *Ann Rheum Dis* 2010, **69**:319-324.
7. Chauhan SK, Tripathy NK, Nityanand S: **Antigenic targets and pathogenicity of anti-aortic endothelial cell antibodies in Takayasu arteritis.** *Arthritis Rheum* 2006, **54**:2326-2333.
8. Del Papa N, Guidali L, Sironi M, Shoenfeld Y, Mantovani A, Tincani A, Balestrieri G, Radice A, Sinico RA, Meroni PL: **Anti-endothelial cell IgG**

antibodies from patients with Wegener's granulomatosis bind to human endothelial cells in vitro and induce adhesion molecule expression and cytokine secretion. *Arthritis Rheum* 1996, **39**:758-766.
9. Ahmed SS, Tan FK, Arnett FC, Jin L, Geng YJ: **Induction of apoptosis and fibrillin 1 expression in human dermal endothelial cells by scleroderma sera containing anti-endothelial cell antibodies.** *Arthritis Rheum* 2006, **54**:2250-2262.
10. Hahn BH: **Targeted therapies in systemic lupus erythematosus: successes, failures and future.** *Ann Rheum Dis* 2011, **70(Suppl 1)**:i64-i66.
11. Bloom O, Cheng KF, He M, Papatheodorou A, Volpe BT, Diamond B, Al-Abed Y: **Generation of a unique small molecule peptidomimetic that neutralizes lupus autoantibody activity.** *Proc Natl Acad Sci USA* 2011, **108**:10255-10259.
12. Youinou P: **New target antigens for anti-endothelial cell antibodies.** *Immunobiology* 2005, **210**:789-797.
13. Naparstek Y, Plotz PH: **The role of autoantibodies in autoimmune disease.** *Annu Rev Immunol* 1993, **11**:79-104.
14. Racanelli V, Prete M, Musaraj G, Dammacco F, Perosa F: **Autoantibodies to intracellular antigens: generation and pathogenetic role.** *Autoimmun Rev* 2011, **10**:503-508.
15. Praprotnik S, Blank M, Meroni PL, Rozman B, Eldor A, Shoenfeld Y: **Classification of anti-endothelial cell antibodies into antibodies against microvascular and macrovascular endothelial cells: the pathogenic and diagnostic implications.** *Arthritis Rheum* 2001, **44**:1484-1494.
16. Karasawa R, Kurokawa MS, Yudoh K, Masuko K, Ozaki S, Kato T: **Peroxiredoxin 2 is a novel autoantigen for anti-endothelial cell antibodies in systemic vasculitis.** *Clin Exp Immunol* 2010, **161**:459-470.
17. Dieudé M, Senécal JL, Raymond Y: **Induction of endothelial cell apoptosis by heat-shock protein 60-reactive antibodies from anti-endothelial cell autoantibody-positive systemic lupus erythematosus patients.** *Arthritis Rheum* 2004, **50**:3221-3231.
18. Kitamura T, Koshino Y, Shibata F, Oki T, Nakajima H, Nosaka T, Kumagai H: **Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics.** *Exp Hematol* 2003, **31**:1007-1014.
19. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF: **The 1982 revised criteria for the classification of systemic lupus erythematosus.** *Arthritis Rheum* 1982, **25**:1271-1277.
20. Alarcón-Segovia D, Cardiel MH: **Comparison between three diagnostic criteria for mixed connective tissue disease: study of 593 patients.** *J Rheumatol* 1989, **16**:328-334.
21. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, Daniels TE, Fox PC, Fox RI, Kassan SS, Pillemer SR, Talal N, Weisman MH: **Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group.** *Ann Rheum Dis* 2002, **61**:554-558.
22. Bohan A, Peter JB: **Polymyositis and dermatomyositis (parts 1 and 2).** *N Engl J Med* 1975, **292**:344-347, 403-407.
23. Subcommittee for Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee: **Preliminary criteria for the classification of systemic sclerosis (scleroderma).** *Arthritis Rheum* 1980, **23**:581-590.
24. Aletaha D, Neogi T, Silman AJ, Funovits J, Felson DT, Bingham CO, Birnbaum NS, Burmester GR, Bykerk VP, Cohen MD, Combe B, Costenbader KH, Dougados M, Emery P, Ferraccioli G, Hazes JM, Hobbs K, Huizinga TW, Kavanaugh A, Kay J, Kvien TK, Laing T, Mease P, Ménard HA, Moreland LW, Naden RL, Pincus T, Smolen JS, Stanislawska-Biernat E, Symmons D, Tak PP, Upchurch KS, Vencovský J, Wolfe F, Hawker G: **2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative.** *Arthritis Rheum* 2010, **62**:2569-2581.
25. Miyakis S, Lockshin MD, Atsumi T, Branch DC, Brey RL, Cervera R, Derksen RH, De Groot PG, Koike T, Meroni PL, Reber G, Shoenfeld Y, Tincani A, Vlachoyiannopoulos PG, Krilis SA: **International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS).** *J Thromb Haemost* 2006, **4**:295-306.
26. Lightfoot RW Jr, Michel BA, Bloch DA, Hunder GG, Zvaifler NJ, McShane DJ, Arend WP, Calabrese LH, Leavitt RY, Lie JT, Masi AT, Mills JA, Stevens MB, Wallace SL: **The American College of Rheumatology 1990 criteria for the classification of polyarteritis nodosa.** *Arthritis Rheum* 1990, **33**:1088-1093.
27. Masi AT, Hunder GG, Lie JT, Michel BA, Bloch DA, Arend WP, Calabrese LH, Edworthy SM, Fauci AS, Leavitt RY, Lightfoot RW Jr, McShane DJ, Mills JA, Stevens MB, Wallace SL, Zvaifler NJ: **The American College of Rheumatology 1990 criteria for the classification of Churg-Strauss syndrome (allergic granulomatosis and angiitis).** *Arthritis Rheum* 1990, **33**:1094-1100.
28. Jennette JC, Falk RJ, Andrassy K, Bacon PA, Churg J, Gross WL, Hagen EC, Hoffman GS, Hunder GG, Kallenberg CG, McCluskey RT, Sinico RA, Rees AJ, Van Es LA, Waldherr R, Wiik A: **Nomenclature of systemic vasculitides: proposal of an international consensus conference.** *Arthritis Rheum* 1994, **37**:187-192.
29. Leavitt RY, Fauci AS, Bloch DA, Michel BA, Hunder GG, Arend WP, Calabrese LH, Fries JF, Lie JT, Lightfoot RE Jr, Masi AT, McShane DJ, Mills JA, Stevens MB, Wallace SL, Zvaifler NJ: **The American College of Rheumatology 1990 criteria for the classification of Wegener's granulomatosis.** *Arthritis Rheum* 1990, **33**:1101-1107.
30. Hunder GG, Bloch DA, Michel BA, Stevens MB, Arend WP, Calabrese LH, Edworthy SM, Fauci AS, Leavitt RY, Lie JT, Lightfoot RW Jr, Masi AT, McShane DJ, Mills JA, Wallace SL, Zvaifler NJ: **The American College of Rheumatology 1990 criteria for the classification of giant cell arteritis.** *Arthritis Rheum* 1990, **33**:1122-1128.
31. Arend WP, Michel BA, Bloch DA, Hunder GG, Calabrese LH, Edworthy SM, Fauci AS, Leavitt RY, Lie JT, Lightfoot RW Jr, Masi AT, McShane DJ, Mills JA, Stevens MB, Wallace SL, Zvaifler NJ: **The American College of Rheumatology 1990 criteria for the classification of Takayasu arteritis.** *Arthritis Rheum* 1990, **33**:1129-1134.
32. Tunç R, Uluhan A, Melikoğlu M, Ozyazgan Y, Ozdoğan H, Yazici H: **A reassessment of the International Study Group criteria for the diagnosis (classification) of Behçet's syndrome.** *Clin Exp Rheumatol* 2001, **19(Suppl)**:45-47.
33. Bordron A, Révélen R, D'Arbonneau F, Dueymes M, Renaudineau Y, Jamin C, Youinou P: **Functional heterogeneity of anti-endothelial cell antibodies.** *Clin Exp Immunol* 2001, **124**:492-501.
34. Morita S, Kojima T, Kitamura T: **Plat-E: an efficient and stable system for transient packaging of retroviruses.** *Gene Ther* 2000, **7**:1063-1066.
35. Tripathy NK, Upadhyaya S, Sinha N, Nityanand S: **Complement and cell mediated cytotoxicity by antiendothelial cell antibodies in Takayasu's arteritis.** *J Rheumatol* 2001, **28**:805-808.
36. Idusogie EE, Presta LG, Gazzano-Santoro H, Totpal K, Wong PY, Ultsch M, Meng YG, Mulkerrin MG: **Mapping of the C1q binding site on rituxan, a chimeric antibody with a human IgG1 Fc.** *J Immunol* 2000, **164**:4178-4184.
37. Yamane-Ohnuki N, Kinoshita S, Inoue-Urakubo M, Kusunoki M, Iida S, Nakano R, Wakitani M, Niwa R, Sakurada M, Uchida K, Shitara K, Satoh M: **Establishment of FUT8 knockout Chinese hamster ovary cells: an ideal host cell line for producing completely defucosylated antibodies with enhanced antibody-dependent cellular cytotoxicity.** *Biotechnol Bioeng* 2004, **87**:614-622.
38. Lacy SE, Bönnemann CG, Buzney EA, Kunkel LM: **Identification of FLRT1, FLRT2, and FLRT3: a novel family of transmembrane leucine-rich repeat proteins.** *Genomics* 1999, **62**:417-426.
39. Suzuki J, Umeda M, Sims PJ, Nagata S: **Calcium-dependent phospholipid scrambling by TMEM16F.** *Nature* 2010, **468**:834-838.
40. Alard JE, Dueymes M, Youinou P, Jamin C: **Modulation of endothelial cell damages by anti-Hsp60 autoantibodies in systemic autoimmune diseases.** *Autoimmun Rev* 2007, **6**:438-443.
41. Jamin C, Dugué C, Alard JE, Jousse S, Saraux A, Guillevin L, Piette JC, Youinou P: **Induction of endothelial cell apoptosis by the binding of anti-endothelial cell antibodies to Hsp60 in vasculitis-associated systemic autoimmune diseases.** *Arthritis Rheum* 2005, **52**:4028-4038.
42. Yamagishi S, Hampel F, Hata K, Del Toro D, Schwark M, Kvachnina E, Bastmeyer M, Yamashita T, Tarabykin V, Klein R, Egea J: **FLRT2 and FLRT3 act as repulsive guidance cues for Unc5-positive neurons.** *EMBO J* 2011, **30**:2920-2933.
43. Wei K, Xu Y, Tse H, Manolson MF, Gong SG: **Mouse FLRT 2 interacts with the extracellular and intracellular regions of FGFR2.** *J Dent Res* 2011, **90**:1234-1239.
44. Xu Y, Wei K, Kulyk W, Gong SG: **FLRT2 promotes cellular proliferation and inhibits cell adhesion during chondrogenesis.** *J Cell Biochem* 2011, **112**:3440-3448.
45. Gong SG, Mai S, Chung K, Wei K: **Flrt2 and Flrt3 have overlapping and non-overlapping expression during craniofacial development.** *Gene Expr Patterns* 2009, **9**:497-502.

46. Müller PS, Schulz R, Maretto S, Costello I, Srinivas S, Bikoff E, Robertson E: **The fibronectin leucine-rich repeat transmembrane protein Flrt2 is required in the epicardium to promote heart morphogenesis.** *Development* 2011, **138**:1297-1308.
47. Leung DY, Moake JL, Havens PL, Kim M, Pober JS: **Lytic anti-endothelial cell antibodies in haemolytic-uraemic syndrome.** *Lancet* 1988, **2**:183-186.
48. Fujieda M, Oishi N, Kurashige T: **Antibodies to endothelial cells in Kawasaki disease lyse endothelial cells without cytokine pretreatment.** *Clin Exp Immunol* 1997, **107**:120-126.
49. Salmon JE, Roman MJ: **Subclinical atherosclerosis in rheumatoid arthritis and systemic lupus erythematosus.** *Am J Med* 2008, **121(Suppl 1)**:3-8.
50. Roman MJ, Shanker BA, Davis A, Lockshin MD, Sammaritano L, Simantov R, Crow MK, Schwartz JE, Paget SA, Devereux RB, Salmon JE: **Prevalence and correlates of accelerated atherosclerosis in systemic lupus erythematosus.** *N Engl J Med* 2003, **349**:2399-2406.

doi:10.1186/ar3897
Cite this article as: Shirai *et al.*: 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. *Arthritis Research & Therapy* 2012 **14**:R157.

A Genome-Wide Association Study Identified *AFF1* as a Susceptibility Locus for Systemic Lupus Eyrthematosus in Japanese

Yukinori Okada[1,2,3☯], Kenichi Shimane[1,2☯], Yuta Kochi[1,2☯*], Tomoko Tahira[4], Akari Suzuki[1], Koichiro Higasa[3], Atsushi Takahashi[3], Tetsuya Horita[5], Tatsuya Atsumi[5], Tomonori Ishii[6], Akiko Okamoto[2], Keishi Fujio[2], Michito Hirakata[7], Hirofumi Amano[8], Yuya Kondo[9], Satoshi Ito[9], Kazuki Takada[10], Akio Mimori[11], Kazuyoshi Saito[12], Makoto Kamachi[13], Yasushi Kawaguchi[14], Katsunori Ikari[14], Osman Wael Mohammed[15], Koichi Matsuda[15], Chikashi Terao[16,17], Koichiro Ohmura[16], Keiko Myouzen[1], Naoya Hosono[18], Tatsuhiko Tsunoda[19], Norihiro Nishimoto[20], Tsuneyo Mimori[16], Fumihiko Matsuda[17], Yoshiya Tanaka[12], Takayuki Sumida[9], Hisashi Yamanaka[14], Yoshinari Takasaki[8], Takao Koike[5], Takahiko Horiuchi[21], Kenshi Hayashi[4], Michiaki Kubo[18], Naoyuki Kamatani[3], Ryo Yamada[1,17], Yusuke Nakamura[15], Kazuhiko Yamamoto[1,2]

1 Laboratory for Autoimmune Diseases, Center for Genomic Medicine (CGM), RIKEN, Yokohama, Japan, **2** Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan, **3** Laboratory for Statistical Analysis, CGM, RIKEN, Yokohama, Japan, **4** Division of Genome Analysis, Research Center for Genetic Information, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan, **5** Department of Medicine II, Hokkaido University Graduate School of Medicine, Sapporo, Japan, **6** Department of Hematology and Rheumatology, Tohoku University Graduate School of Medicine, Sendai, Japan, **7** Division of Rheumatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan, **8** Department of Internal Medicine and Rheumatology, Juntendo University School of Medicine, Tokyo, Japan, **9** Division of Clinical Immunology, Doctoral Program in Clinical Sciences, Graduate School of Comprehensive Human Science, University of Tsukuba, Tsukuba, Japan, **10** Departments of Medicine and Rheumatology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan, **11** Division of Rheumatic Diseases, National Center for Global Health and Medicine, Tokyo, Japan, **12** First Department of Internal Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan, **13** Department of Immunology and Rheumatology, Unit of Translational Medicine, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan, **14** Institute of Rheumatology, Tokyo Women's Medical University, Tokyo, Japan, **15** Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan, **16** Department of Rheumatology and Clinical immunology, Graduate School of Medicine, Kyoto University, Kyoto, Japan, **17** Center for Genomic Medicine, Kyoto University Graduate School of Medicine, Kyoto, Japan, **18** Laboratory for Genotyping Development, CGM, RIKEN, Yokohama, Japan, **19** Laboratory for Medical Informatics, CGM, RIKEN, Yokohama, Japan, **20** Laboratory of Immune Regulation, Wakayama Medical University, Wakayama, Japan, **21** Department of Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan

Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease that causes multiple organ damage. Although recent genome-wide association studies (GWAS) have contributed to discovery of SLE susceptibility genes, few studies has been performed in Asian populations. Here, we report a GWAS for SLE examining 891 SLE cases and 3,384 controls and multi-stage replication studies examining 1,387 SLE cases and 28,564 controls in Japanese subjects. Considering that expression quantitative trait loci (eQTLs) have been implicated in genetic risks for autoimmune diseases, we integrated an eQTL study into the results of the GWAS. We observed enrichments of cis-eQTL positive loci among the known SLE susceptibility loci (30.8%) compared to the genome-wide SNPs (6.9%). In addition, we identified a novel association of a variant in the AF4/FMR2 family, member 1 (*AFF1*) gene at 4q21 with SLE susceptibility (rs340630; $P=8.3\times10^{-9}$, odds ratio = 1.21). The risk A allele of rs340630 demonstrated a cis-eQTL effect on the *AFF1* transcript with enhanced expression levels ($P<0.05$). As *AFF1* transcripts were prominently expressed in $CD4^+$ and $CD19^+$ peripheral blood lymphocytes, up-regulation of *AFF1* may cause the abnormality in these lymphocytes, leading to disease onset.

Citation: Okada Y, Shimane K, Kochi Y, Tahira T, Suzuki A, et al. (2012) A Genome-Wide Association Study Identified *AFF1* as a Susceptibility Locus for Systemic Lupus Eyrthematosus in Japanese. PLoS Genet 8(1): e1002455. doi:10.1371/journal.pgen.1002455

Editor: Mark I. McCarthy, University of Oxford, United Kingdom

Received May 16, 2011; **Accepted** November 18, 2011; **Published** January 26, 2012

Funding: This study was supported by a grant from the CGM, RIKEN, and a grant from the autoimmune disease study group of Research in Intractable Diseases, Japanese Ministry of Health, Labor, and Welfare, Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: ykochi@src.riken.jp

☯ These authors contributed equally to this work.

Author Summary

Although recent genome-wide association study (GWAS) approaches have successfully contributed to disease gene discovery, many susceptibility loci are known to be still uncaptured due to strict significance threshold for multiple hypothesis testing. Therefore, prioritization of GWAS results by incorporating additional information is recommended. Systemic lupus erythematosus (SLE) is an autoimmune disease that causes multiple organ damage. Considering that abnormalities in B cell activity play essential roles in SLE, prioritization based on an expression quantitative trait loci (eQTLs) study for B cells would be a promising approach. In this study, we report a GWAS and multi-stage replication studies for SLE examining 2,278 SLE cases and 31,948 controls in Japanese subjects. We integrated eQTL study into the results of the GWAS and identified *AFF1* as a novel SLE susceptibility loci. We also confirmed cis-regulatory effect of the locus on the *AFF1* transcript. Our study would be one of the initial successes for detecting novel genetic locus using the eQTL study, and it should contribute to our understanding of the genetic loci being uncaptured by standard GWAS approaches.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by autoantibody production, complement activation, and multi-organ damage [1]. Familial aggregation demonstrates that both genetic and environmental factors play a role in pathogenesis of SLE [2]. Genetic studies using candidate gene-approaches, and recently, genome-wide association studies (GWAS), have uncovered more than 25 SLE susceptibility genes, including *HLA-DRB1*, *IRF5*, *STAT4*, *ITGAM*, *BLK*, *TNFAIP3*, and others [3–18]. However, most of these studies were conducted in European populations [3–13,15,17], and few studies have been conducted in Asian populations [14,16,18]. Since the epidemiology of SLE has demonstrated that the prevalence of disease substantially differs among populations, genetic backgrounds of SLE should be also heterogeneous across populations [19,20]. Therefore, additional studies in Asians might provide novel insights. It is of note that GWAS for SLE in Chinese populations identified novel loci that had not been detected in Europeans, such as *ETS1*, *IKZF1*, and *WDFY4* [14,16].

Another issue raised by the previous GWASs for complex diseases is that many susceptibility loci still remained uncaptured, owing to its strict significance threshold for multiple hypothesis testing [21]. In SLE, for example, the 26 risk loci identified by the previous GWAS explained only an estimated 8% of the total genetic susceptibility to the disease [15]. Therefore, it is still important to examine the sub-loci of GWAS, in order to reveal the entire picture of genetic etiology. To effectively explore these uncaptured loci, prioritization of GWAS results by incorporating additional information implicated in the disease pathophysiology is recommended [22,23]. Considering that abnormalities in B cell activity play essential roles in SLE [1] and that expression quantitative trait loci (eQTL) have been implicated to comprise approximately a half of genetic risks for autoimmune diseases [24], prioritization based on an eQTL study for B cells would be a promising approach for SLE [25]. Moreover, an eQTL itself assures the presence of functional variant(s) that regulate gene expression. Thus, eQTL increases the prior probability of the presence of disease-causal variant(s) in the locus more effectively and unbiasedly, compared to other knowledge-based prioritizations such as gene pathway analysis [24].

Here, we report a GWAS and multi-stage replication studies for SLE examining 2,278 SLE cases and 31,948 controls in Japanese subjects. We integrated eQTL study into the results of the GWAS, which effectively enabled to detect a novel SLE susceptibility locus.

Results

GWAS for SLE

In the GWAS, 891 SLE cases and 3,384 controls in Japanese subjects were genotyped over 550,000 single nucleotide polymorphism (SNP) markers (Table S1, S2 and Figure 1). We applied stringent quality control (QC) criteria and evaluated associations of 430,797 autosomal SNPs, as previously described [26]. No substantial population stratification was demonstrated through principal component analysis (Figure S1) or a Quantile–Quantile plot of *P*-values (inflation factor, $\lambda_{GC,} = 1.088$, Figure S2), suggesting homogenous ancestries of our study population [27].

We identified significant associations in six chromosomal loci that satisfied the genome-wide significance threshold of $P<5.0\times10^{-8}$ (Table 1 and Figure 2A). These loci have been reported to be associated with SLE susceptibility (*STAT4*, *TNFAIP3*, *HIP1*, *BLK*, *ETS1*, and the HLA region) [3–18]. We also observed significant replications in 17 of the previously reported SLE susceptibility loci [3–18] ($\alpha = 0.01$; Table 2). Of these, significant replications were enriched in the loci identified through the studies in Asian populations (80%; 8 of the 10 loci), including *RASGRP3*, *IKZF1*, *HIP1*, *WDFY4*, intergenic region at 11q23, *ETS1*, *SLC15A4*, *ELF1*, and *HIC2-UBE2L3* [14,16,18], compared to those in European populations (56.3%; 9 of the 16 loci) [3–13,15,17].

Incorporation of eQTL study into GWAS results

For the selection of SNPs incorporated in the replication studies of the potential association signals, we evaluated cis-eQTL effects of the SNPs using publically available gene expression data [28], and prioritized the results of the GWAS. After applying QC criteria, we evaluated the expression levels of 19,047 probes assayed in lymphoblastoid B cell lines from Phase II HapMap East-Asian individuals [29] using Illumina's human whole-genome expression array (WG-6 version 1) [28]. For each of the SNPs included in our GWAS, probes located within ±300 kbp regions were focused on as cis-eQTLs (average 4.93 probes per SNP). We denoted the SNPs which exhibited significant associations with expression levels of any of the corresponding cis-eQTLs as eQTL positive (false discovery rate (FDR) *Q*-values<0.2). We observed enrichments of eQTL positive loci among the SLE susceptibility loci (30.8%; 8 of the 26 evaluated loci) including a well-known eQTL gene of *BLK* [11,25] (Table 2), compared to the genome-wide SNPs (6.9%) and compared even to the SNPs in the vicinity of expressed loci (among the SNPs located within ±10 kbp of probes used for the expression analysis, 13.1% were eQTL positive; Table S3).

By prioritizing the results of the GWAS using the eQTL study, we selected 57 SNPs from 1,207 SNPs that satisfied $P<1.0\times10^{-3}$ in the GWAS. We subsequently referred the associations of the selected SNPs using the results of the concurrent genome-wide scan for SLE in an independent Japanese population (Tahira T et al. Presented at the 59th Annual Meeting of the American Society of Human Genetics, October 21, 2009). In the scan, 447 SLE cases and 680 controls of Japanese origin were evaluated using a pooled DNA approach [30]. We selected SNPs if any association signals were observed in the neighboring SNPs of the

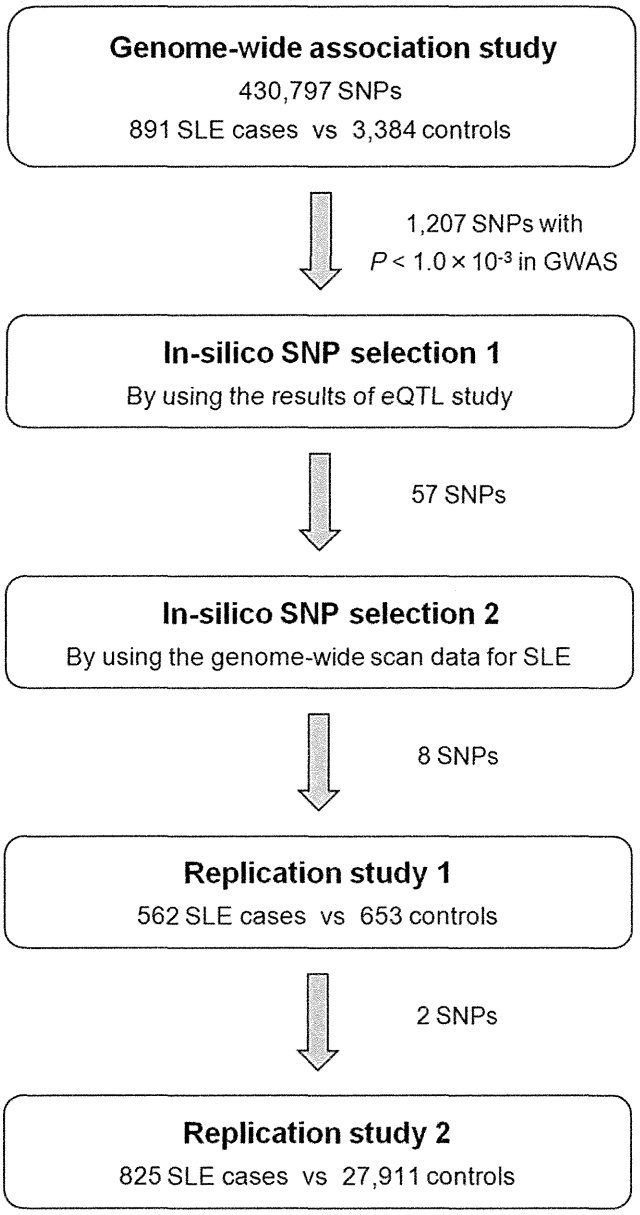

Figure 1. Design of the GWAS and multi-stage replication studies for SLE in Japanese subjects. A total of 2,278 SLE cases and 31,948 controls were enrolled. The clinical characteristics of the subjects are summarized in Table S1 and S2. Details of the genome-wide scan data for SLE referenced in the *in silico* SNP selection 2 are described elsewhere (Tahira T et al. Presented at the 59th Annual Meeting of the American Society of Human Genetics, October 21, 2009).
doi:10.1371/journal.pgen.1002455.g001

pooled analysis. As a result, 8 SNPs remained for further investigation (Table S4).

Replication studies and identification of *AFF1*

Then, we performed two-stage replication studies using independent SLE cohorts for Japanese subjects (cohort 1 with 562 SLE cases and 653 controls, and cohort 2 with 825 SLE cases and 27,911 controls). First, we evaluated the selected 8 SNPs in the replication study 1. In the replication study 2, 2 SNPs that satisfied $P<1.0\times10^{-6}$ in the combined study of GWAS and replication study 1 were further evaluated (Figure 1). Among the evaluated SNPs, we observed significant replications in the SNP located in the genomic region of the AF4/FMR2 family, member 1 gene (*AFF1*) at 4q21 (rs340630; $P=4.6\times10^{-5}$ and $P=0.0094$ in the two individual cohorts, respectively; Table 3, Table S5, and Figure 2B). The combined study for the GWAS ($P=1.5\times10^{-4}$) and the replication studies demonstrated significant associations of rs340630 that satisfied the genome-wide significance threshold ($P=8.3\times10^{-9}$, OR = 1.21, 95% CI 1.14–2.30).

Cis-eQTL effect of rs340630 on *AFF1* transcripts

Since the landmark SNP in the *AFF1* locus, rs340630, was prioritized through the eQTL study as an eQTL positive SNP (Table 3), we further validated its cis-eQTL effect using Epstein-Barr virus (EBV)-transfected B cell lines established from Japanese individuals (Pharma SNP Consortium (PSC) cells, $n=62$). The correlation between rs340630 genotypes and the expression levels of *AFF1* was significant in the PSC cells stimulated with phorbol myristate acetate (PMA) ($R^2=0.074$, $P=0.033$; Figure 3A). The expression levels increased with the number of SLE-risk (A) alleles. To further confirm this cis-regulatory effect, we performed allele-specific transcript quantification (ASTQ) of *AFF1*. The transcript levels of each allele were quantified by qPCR using an allele specific probe for a SNP in the 5′-untranslated region (rs340638), which was in absolute LD with rs340630 ($r^2=1.0$, $D'=1.0$). We examined PSC-cells ($n=17$) that were heterozygous for both rs340630 and rs340638. The mean ratio of each transcript (A over G allele; the A allele comprises a haplotype with the risk (A) allele of rs340630) were significantly increased to 1.07 compared to the ratio of the amount of DNA (1.00, $P=0.012$) (Figure 3B). These results suggest that rs340630, or SNP(s) in LD with it, are a regulatory variant predisposing SLE susceptibility through increased expression levels of *AFF1*.

Expression of *AFF1* in $CD4^+$ and $CD19^+$ peripheral blood lymphocytes

AFF1 is known to be involved in cytogenetic translocations of acute lymphoblastic leukemia (ALL) [31]. Its fusion protein with the mixed-lineage leukemia gene (*MLL*) is implicated in the regulation of transcription and the cell cycle of lymphocytes [31]. To investigate the expression pattern of *AFF1* in normal tissues, we evaluated the transcript levels of *AFF1* in a panel of various tissues. We observed prominent expression of *AFF1* in $CD4^+$ and $CD19^+$ peripheral blood lymphocytes, implying an important role for *AFF1* in helper-T-cells and B-cells (Figure 3C).

Discussion

Through a GWAS and multi-staged replication studies consisting of 2,278 SLE cases and 31,948 controls in Japanese subjects, our study identified that the *AFF1* locus was significantly associated with SLE susceptibility.

As well as the identification of the novel SLE susceptibility locus, we observed significant replications of associations in the previously reported susceptibility loci. The replications were especially enriched in the loci identified through the studies in Asian populations, compared to those in European populations. Considering the ethnical heterogeneities in the epidemiology of SLE [19,20], these observations suggest the similarities in the genetic backgrounds of SLE shared within Asian populations, and also the existence of the both common and divergent genetic backgrounds encompassed between European and Asian populations.

Table 1. Results of a genome-wide association study for Japanese patients with SLE.

rsID[a]	Chr	Position (bp)	Cytoband	Gene	Allele[b]	No. subjects		Allele 1 freq.		OR (95%CI)	*P*
					1/2	Case	Control	Case	Control		
rs10168266	2	191,644,049	2q32	*STAT4*	T/C	891	3,384	0.37	0.27	1.59 (1.42–1.78)	2.7×10^{-16}
rs9501626	6	32,508,322	6p21	HLA region	A/C	891	3,381	0.20	0.12	1.86 (1.62–2.13)	1.0×10^{-18}
rs2230926	6	138,237,759	6q23	*TNFAIP3*	G/T	891	3,377	0.11	0.069	1.75 (1.47–2.08)	1.9×10^{-10}
rs6964720	7	75,018,280	7q11	*HIP1*	G/A	891	3,384	0.25	0.19	1.43 (1.27–1.63)	1.3×10^{-8}
rs2254546	8	11,381,089	8p23	*BLK*	G/A	891	3,384	0.78	0.72	1.42 (1.61–1.25)	4.1×10^{-8}
rs6590330	11	127,816,269	11q24	*ETS1*	A/G	891	3,368	0.48	0.39	1.44 (1.30–1.60)	1.3×10^{-11}

[a]SNPs that satisfied the threshold of $P<5.0\times10^{-8}$ were indicated.
[b]Based on forward strand of NCBI Build 36.3.
SLE, systemic lupus erythematosus; OR, odds ratio.
doi:10.1371/journal.pgen.1002455.t001

To effectively detect the novel SLE susceptibility locus, we integrated cis-eQTL effects of the SNPs and prioritized the results of the GWAS. In addition to identifying a novel locus for SLE-susceptibility, our study demonstrated approximately 30% of confirmed SLE-susceptibility loci were comprised of cis-eQTLs. We also confirmed cis-regulatory effect of the landmark SNP in the *AFF1* locus, rs340630, on *AFF1* transcripts, which had been prioritized through the eQTL study. These results would suggest that accumulation of quantitative changes in gene expression would accelerate the disease onset of SLE. It would also demonstrate the validity of applying eQTL study in the search of the susceptible genes for SLE or other autoimmune diseases, as previously suggested in the study for celiac disease [24]. To our knowledge, this is one of the initial studies to successfully discover a new locus by prioritizing GWAS results using eQTLs, and should contribute to the approaches assessing genetic loci still being uncaptured by recent large-scaled GWASs due to stringent significance threshold for multiple hypothesis testing [21].

We observed prominent expression levels of *AFF1* in $CD4^+$ and $CD19^+$ peripheral blood lymphocytes, which would imply an important role for *AFF1* in helper-T-cells and B-cells. In fact, *AFF1* is essential for normal lymphocyte development, as demonstrated in mice deficient for *AFF1*; severe reduction were observed in the thymic double positive CD4/CD8 population and the bone marrow pre-B and mature B-cell numbers [32]. The risk A allele of rs340630 demonstrated a cis-eQTL effect on the *AFF1* transcript with enhanced expression levels. As the *AFF1* locus was also demonstrated as an eQTL in primary liver cells [33], the cis-regulatory effect may hold in primary cells as well as lymphoblastoid cells used in the present study. However, because the mechanism of transcriptional regulation is substantially different among cell types [34], cell-type specific analyses including those for primary T-cells and B-cells are needed for understanding the precise role of *AFF1* variant in primary lymphocytes. Although further functional investigation is necessary, our observation suggested that *AFF1* is involved in the etiology of SLE through the regulation of development and activity of lymphocytes. It is of note that *AFF3*, which also belongs to the AF4/FMR2 family, is associated with susceptibility to autoimmune diseases [35].

One of our study's limitations is the selection of SNPs for the replication study using the results of the pooled DNA approach [30], which used a different genotyping platform from that of the present GWAS. Moreover, the association signals based on Silhouette scores in pooled analysis would be less reliable compared to those based on individual genotyping. Since direct comparisons of the association signals of the same single SNPs between the studies would be difficult due to these issues, we adopted the complementary approach that referred the association signals of the multiple SNPs in the pooled analysis for each of the single SNPs in the GWAS, taking account of LD and physical distances between the SNPs. However, there would exist a possibility that the variant(s) truly associated with SLE was left not to be examined in the replication study. It should be noted that only 1 SNP among the 8 selected SNPs yielded the significant association with SLE, although further enrichments of the significant associations might be anticipated. To elucidate effectiveness and limitation of our approach, further assessments of the studies on the remaining loci would be desirable. It should also be noted that the control-case ratio of the subjects were relatively high in the replication study 2 (= 33.8), and this disproportionate ratio could have induced potential bias on the results of the association analysis of the SNPs. However, considering the homogeneous ancestries of the Japanese population [27] and that principal component analysis did not demonstrate significant population stratification in the control subjects of the replication study 2 (data not shown), the bias owing to population stratification might not be substantial.

In summary, through a GWAS and multi-staged replication studies in a Japanese population integrating eQTL study, our study identified *AFF1* as a novel susceptibility locus for SLE.

Materials and Methods

Subjects

We enrolled 2,278 systemic lupus erythematosus (SLE) cases and 31,948 controls. SLE cases enrolled in the genome-wide association study (GWAS) ($n = 891$) or part of the 2nd replication study ($n = 83$) were collected from 12 medical institutes in Japan under the support of the autoimmune disease study group of Research in Intractable Diseases, Japanese Ministry of Health, Labor and Welfare: Hokkaido University Graduate School of Medicine, Tohoku University Graduate School of Medicine, the University of Tokyo, Keio University School of Medicine, Juntendo University School of Medicine, University of Occupational and Environmental Health, University of Tsukuba, Tokyo Medical and Dental University, National Center for Global Health and Medicine, Nagasaki University, Wakayama Medical University, and Jichi Medical University. SLE cases ($n = 562$) and controls ($n = 653$) enrolled in the 1st replication study were collected from Kyushu University. Some of the SLE cases ($n = 742$) and controls ($n = 27{,}911$) enrolled in the 2nd replication study were collected from Kyoto University, Tokyo Women's

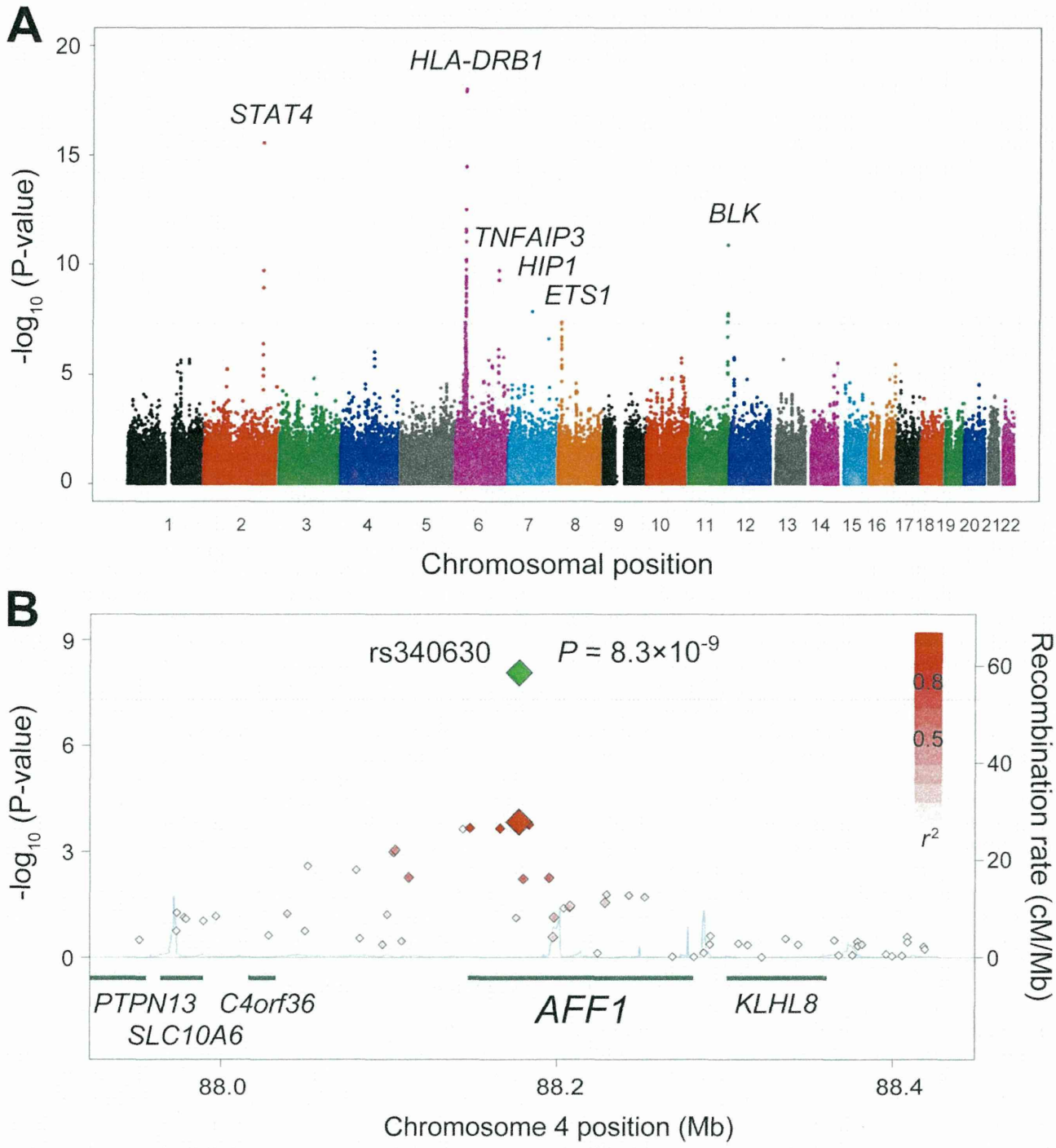

Figure 2. Associations of the *AFF1* locus with SLE. (A) A chromosomal plot of *P*-values in GWAS for SLE. (B) A regional plot in the *AFF1* locus. Diamond-shaped data points represent $-\log_{10}$ (*P*-values) of the SNPs. Large-sized points indicate the *P*-values of the landmark SNP, rs340630 (green for the combined study and red for the GWAS). Density of red color represents r^2 values with rs340630. Blue line represents recombination rates. Lower part indicates RefSeq genes. Gray dashed horizontal lines represent the threshold of $P = 5.0 \times 10^{-8}$. The plots were drawn using SNAP, version 2.1 [47].
doi:10.1371/journal.pgen.1002455.g002

Medical University, the University of Tokyo, and the BioBank Japan Project [36]. All subjects were of Japanese origin and provided written informed consent. SLE cases met the revised American College of Rheumatology (ACR) criteria for SLE [37]. Control subjects were confirmed to be free of autoimmune disease. Some of the SLE cases were included in our previous studies [38–40]. Details of the subjects are summarized in Table S1 and S2. This research project was approved by the ethical committees of the University of Tokyo, RIKEN, and affiliated medical institutes.

Table 2. Associations among previously reported SLE-related loci.

rsID	Chr	Position (bp)	Cytoband	Gene	Allele[a]	Allele 1 freq.		OR (95%CI)	*P*	eQTL[b]	Identified by the studies in[c]	
					1/2	Case	Control				Caucasians	Asians
rs2205960	1	171,458,098	1q25	*TNFSF4*	T/G	0.23	0.18	1.35 (1.19–1.54)	3.0×10^{-6}		+	
rs3024505	1	205,006,527	1q32	*IL10*	A/G	0.019	0.014	1.34 (0.90–2.00)	0.15		+	
rs13385731	2	33,555,394	2p22	*RASGRP3*	C/T	0.90	0.87	1.37 (1.15–1.64)	6.0×10^{-4}	+		+
rs10168266	2	191,644,049	2q32	*STAT4*	T/C	0.37	0.27	1.59 (1.42–1.78)	2.7×10^{-16}		+	
rs6445975	3	58,345,217	3p14	*PXK*	G/T	0.25	0.23	1.09 (0.96–1.23)	0.18	+	+	
rs10516487	4	102,970,099	4q24	*BANK1*	G/A	0.91	0.89	1.28 (1.07–1.53)	0.0070		+	
rs10036748	5	150,438,339	5q33	*TNIP1*	T/C	0.75	0.72	1.16 (1.03–1.31)	0.014			+
rs9501626	6	32,508,322	6p21	*HLA-DRB1*	A/C	0.20	0.12	1.86 (1.62–2.13)	1.0×10^{-18}		+	
rs548234	6	106,674,727	6q21	*PRDM1*	C/T	0.40	0.34	1.30 (1.16–1.44)	2.3×10^{-6}	+	+	
rs2230926	6	138,237,759	6q23	*TNFAIP3*	G/T	0.11	0.069	1.75 (1.47–2.08)	1.9×10^{-10}	+	+	
rs849142	7	28,152,416	7p15	*JAZF1*	C/T	0.999	0.999	2.72 (0.25–29.8)	0.41		+	
rs4917014	7	50,276,409	7p12	*IKZF1*	T/G	0.58	0.53	1.24 (1.11–1.38)	8.1×10^{-5}			+
rs6964720	7	75,018,280	7q11	*HIP1*	G/A	0.25	0.19	1.43 (1.27–1.62)	1.3×10^{-8}			+
rs4728142	7	128,361,203	7q32	*IRF5*	A/G	0.16	0.11	1.48 (1.28–1.72)	2.4×10^{-7}	+	+	
rs2254546	8	11,381,089	8p23	*BLK*	G/A	0.78	0.72	1.42 (1.25–1.61)	4.1×10^{-8}	+	+	
rs1913517	10	49,789,060	10q11	*WDFY4*	A/G	0.32	0.28	1.20 (1.07–1.35)	0.0013			+
rs4963128	11	579,564	11p15	*KIAA1542*	T/C	0.98	0.97	1.58 (1.03–2.44)	0.038	+	+	
rs2732552	11	35,041,168	11p13	*PDHX, CD44*	T/C	0.75	0.73	1.13 (1.00–1.27)	0.056		+	
rs4639966	11	118,078,729	11q23	Intergenic	T/C	0.32	0.28	1.22 (1.09–1.36)	7.3×10^{-4}			+
rs6590330	11	127,816,269	11q24	*ETS1*	A/G	0.48	0.39	1.44 (1.30–1.60)	1.3×10^{-11}			+
rs1385374	12	127,866,647	12q24	*SLC15A4*	T/C	0.19	0.16	1.21 (1.06–1.38)	0.0057			+
rs7329174	13	40,456,110	13q14	*ELF1*	G/A	0.30	0.25	1.32 (1.18–1.49)	2.2×10^{-6}			+
rs7197475	16	30,550,368	16p11	Intergenic	T/C	0.12	0.10	1.20 (1.02–0.41)	0.031			+
rs11150610	16	31,241,737	16p11	*ITGAM*	C/A	0.20	0.19	1.07 (0.94–1.22)	0.32	+	+	
rs12949531	17	13,674,531	17p12	Intergenic	T/C	0.28	0.27	1.02 (0.91–1.15)	0.73		+	
rs463426	22	20,139,185	22q11	*HIC2,UBE2L3*	T/C	0.52	0.48	1.20 (1.08–1.33)	6.1×10^{-4}		+	

[a]Based on forward strand of NCBI Build 36.3.
[b]Defined using gene expression data measured in lymphoblastoid B cell lines [28].
[c]Based on the previously reported studies for SLE susceptibility loci [3–18].
SLE, systemic lupus erythematosus; OR, odds ratio; eQTL, expression quantitative trait locus; GWAS, genome-wide association study.
doi:10.1371/journal.pgen.1002455.t002

Genotyping and quality control

In GWAS, 946 SLE cases and 3,477 controls were genotyped using Illumina HumanHap610-Quad and Illumina HumanHap550v3 Genotyping BeadChips (Illumina, CA, USA), respectively. After the exclusion of 47 SLE cases and 92 controls with call rates <0.98, SNPs with call rates <0.99 in SLE cases or controls,

Table 3. Results of combined study for Japanese patients with SLE.

rsID	Chr	Position (bp)	Cytoband	Gene	Allele	Stage	No. subjects		Allele 1 freq.		OR (95%CI)	*P*	eQTL[a]
					1/2		Case	Control	Case	Control			
rs340630	4	88,177,419	4q21	*AFF1*	A/G	GWAS	891	3,383	0.56	0.51	1.22 (1.10–1.36)	1.5×10^{-4}	+
						Replication study 1	550	646	0.57	0.49	1.40 (1.19–1.64)	4.6×10^{-5}	
						Replication study 2	820	27,911	0.56	0.53	1.14 (1.03–1.26)	0.0094	
						Combined study	2,261	31,940	0.56	0.52	1.21 (1.14–1.30)	8.3×10^{-9}	

[a]Defined using gene expression data measured in lymphoblastoid B cell lines [28].
doi:10.1371/journal.pgen.1002455.t003

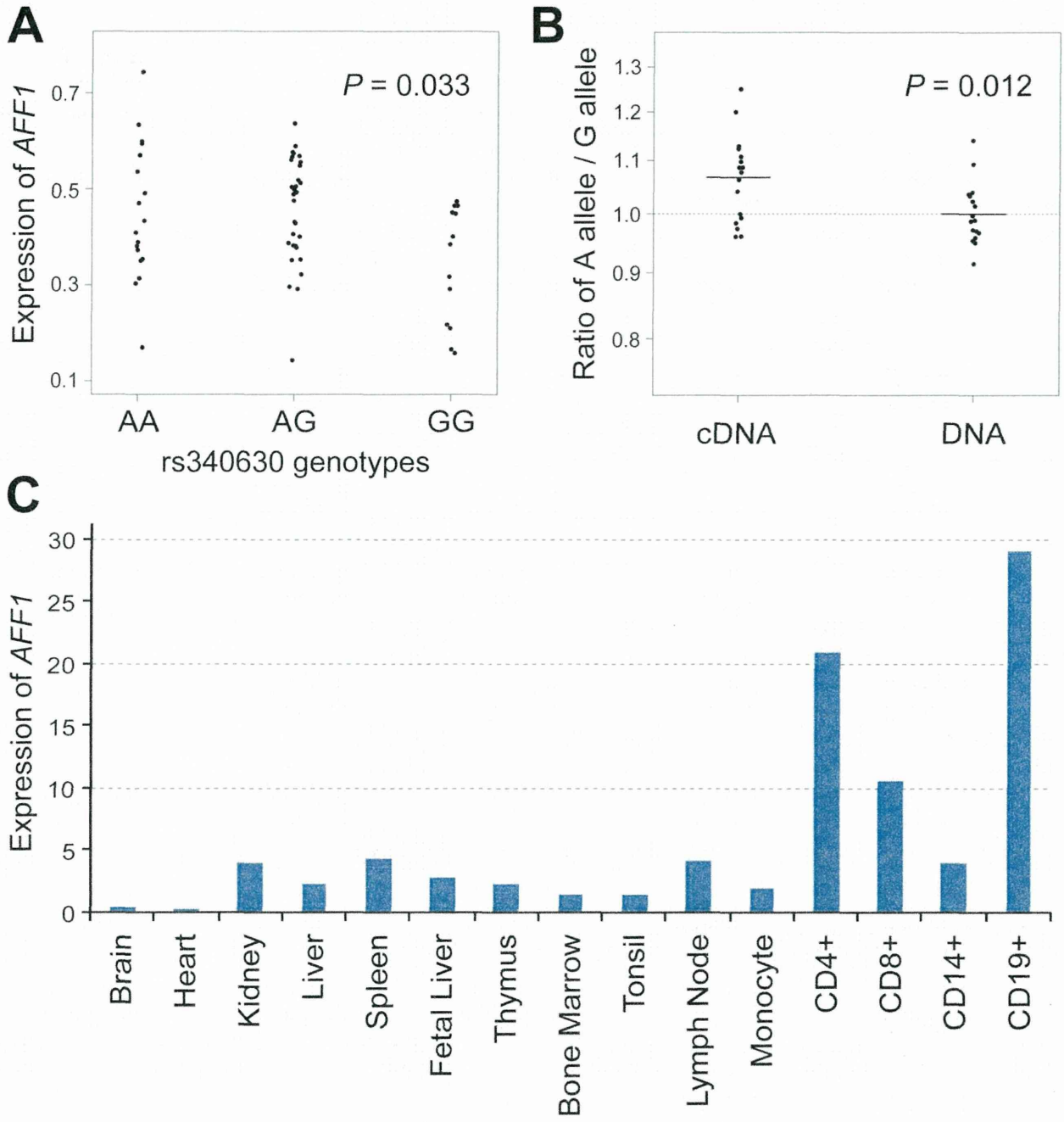

Figure 3. Association of rs340630 with *AFF1* expression. (A) Correlation between rs340630 genotypes and transcript levels of *AFF1* (NM_001166693) in EBV-transfected cell lines ($n = 62$) stimulated with PMA. (B) Allele-specific quantification (ASTQ) of *AFF1* transcripts. Allele specific-probes for rs340638 were used for quantification by qPCR. The ratios of A allele over G allele for the amounts of both cDNAs and DNAs were plotted in log scale for each cell line. (C) *AFF1* expression in various tissues. Transcripts levels of *AFF1* were quantified by qPCR and were normalized by *GAPDH* levels.
doi:10.1371/journal.pgen.1002455.g003

non-autosomal SNPs, and SNPs not shared between SLE cases and controls, were excluded. We excluded 7 closely related SLE cases in a 1st or 2nd degree of kinship based on identity-by-descent estimated using PLINK version 1.06 [41]. We then excluded 1 SLE cases and 1 controls whose ancestries were estimated to be distinct from East-Asian populations using PCA performed along with the genotype data of Phase II HapMap populations (release 24) [29] using EIGENSTRAT version 2.0 [42]. Subsequently, SNPs with minor allele frequencies <0.01 in SLE cases or controls, SNPs with exact *P*-values of Hardy-Weinberg equilibrium test $<1.0\times10^{-6}$ in controls, or SNPs with ambiguous cluster plots were excluded. Finally, 430,797 SNPs for 891 SLE cases and 3,384 controls were obtained. Genotyping of SNPs in replication studies was performed using TaqMan Assay or Illumina HumanHap610-Quad Genotyping BeadChip (Illumina, CA, USA).

Association analysis of the SNPs

Association of SNPs in GWAS and replication studies were tested with Cochran-Armitage's trend test. Combined analysis was performed with Mantel-Haenzel method. Associations of previously reported SLE susceptibility loci [3–18] were evaluated using the results of the GWAS. Genotype imputation was performed for non-genotyped SNPs using MACH version 1.0 [43] with Phase II HapMap East-Asian individuals as references [29], as previously described [44]. All imputed SNPs demonstrated imputation scores, *Rsq*, >0.70.

eQTL study

We analyzed gene expression data previously measured in lymphoblastoid B cell lines from Phase II HapMap East-Asian individuals using Illumina's human whole-genome expression array (WG-6 version 1) (accession number; GSE6536) [28]. Expression data were normalized across the individuals. We used BLAST to map 47,294 Illumina array probes onto human autosomal reference genome sequences (Build 36). We discarded probes mapped with expectation values smaller than 0.01 to multiple loci, or for which there was polymorphic HapMap SNP(s) inside the probe. Then, 19,047 probes with exact matches to a unique locus with 100% identity and with a mean signal intensity greater than background were obtained. Genotype data of HapMap individuals were obtained for SNPs included in the GWAS. Associations of SNP genotypes (coded as 0, 1, and 2) with expression levels of each of the cis-eQTL probes (located within ±300 kbp regions of the SNPs) were evaluated using linear regression assuming additive effects of the genotypes on the expression levels. Considering the significant overlap between eQTL and genetic loci responsible for autoimmune diseases [24], we applied relatively less stringent multiple testing threshold of FDR *Q*-values<0.2 for the definition of eQTL. SNPs that exhibited this threshold with any of the corresponding cis-eQTL probes were denoted as eQTL positive.

Selection of SNPs enrolled in the replication studies

In order to select SNPs for further replication studies, we firstly integrated the results of GWAS and eQTL study. SNPs that satisfied $P<1.0\times10^{-4}$ in GWAS, or the SNPs that satisfied $1.0\times10^{-4}\leq P<1.0\times10^{-3}$ in GWAS and denoted as eQTL positive, were selected. Among these, SNPs most significantly associated in each of the genomic loci and not included in the previously reported SLE susceptibility loci [3–18] were further evaluated.

Then, the results of the concurrently proceeding genome-wide scan for SLE in the Japanese subjects using a pooled DNA approach were referred (Tahira T et al. Presented at the 59th Annual Meeting of the American Society of Human Genetics, October 21, 2009). In the scan, DNA collected from 447 SLE cases and 680 controls of Japanese origin were pooled respectively, and genotyped using GeneChip Human Mapping 500K Array Set (Affymetrix, CA, USA). SNPs were ranked according to the Silhouette scores estimated based on relative allele scores (RAS) between SLE cases and controls, and rank-based *P*-values were assigned [30]. By referring to association signals in multiple neighboring SNPs in the pooled analysis, we selected SNPs for replication study 1. Namely, if the SNP of interest was in LD ($r^2>0.5$) or was located within ±100 kbp of SNPs showing association signals in the pooled analysis (rank-based $P<0.01$), it would be selected. SNPs that satisfied $P<1.0\times10^{-6}$ in the combined study of GWAS and replication study 1 were further evaluated in replication study 2 (Figure 1).

Quantification of *AFF1* expression

EBV-transformed lymphoblastoid cell lines ($n=62$) were established by Pharma SNP Consortium (Tokyo, Japan) using peripheral blood lymphocytes of Japanese healthy individuals. Cells were incubated for 2 h in medium alone (RPMI 1640 medium containing 10% FBS, 1% penicillin, and 1% streptomycin) or with 100 ng/ml PMA. Conditions for cell stimulation were optimized before the experiment as previously described [45]. Cells were then harvested and total RNA was isolated using an RNeasy Mini Kit (Qiagen) with DNase treatment. Total RNA (1 μg) was reverse transcribed using TaqMan Gold RT-PCR reagents with random hexamers (Applied Biosystems). Real-time quantitative PCR was performed in triplicate using an ABI PRISM 7900 and TaqMan gene expression assays (Applied Biosystems). Specific probes (Hs01089428_m1) for transcript of *AFF1* (NM_001166693) were used. Expression of *AFF1* in various tissues was also quantified using Premium Total RNA (Clontech). The data were normalized to *GAPDH* levels. *GUS* levels were also evaluated for internal control, and similar results were obtained. Correlation coefficient, R^2, between rs340630 genotypes and transcript levels of *AFF1* was evaluated.

Allele-specific transcript quantification (ASTQ)

ASTQ of *AFF1* in PSC cells was performed as previously described [46]. DNAs were extracted by using a DNeasy Kit (QIAGEN). RNA extraction and cDNA preparation were performed as described above. For PSC cells ($n=17$) that were heterozygous for both rs340630 (the landmark SNP of GWAS) and rs340638 (located in the 5′-untranslated region of *AFF1* and in absolute LD with rs340630), expression levels of *AFF1* were quantified by qPCR on an ABI Prism 7900 using a custom-made TaqMan MGB-probe set for rs340638. Primer sequences were 5′-CTAACTGTGGCCCGCGTTG-3′ and 5′-CCCGGCGCA-GTTTCTGAG-3′. The probe sequences were 5′-VIC-CGAA-GACCGCCAGCGCCCAAC-TAMRA-3′ and 5′-FAM-CGAA-GACCGCCGGCGCCCAA-TAMRA-3′. Ct values of VIC and FAM were obtained for genomic DNA and cDNA samples after 40 cycles of real-time PCR. We also prepared genomic DNA of samples homozygous for each allele and mixed them at different ratios (2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2) to create a standard curve by plotting Ct values of VIC/FAM against the allelic ratio of VIC/FAM for each mixture. Using the standard curve, we calculated the allelic ratios for each genomic DNA and cDNA samples. We measured each sample in quadruplicate in one assay; tests were independently repeated twice.

Web resources

The URLs for data presented herein are as follows.

NCBI GEO, http://www.ncbi.nlm.nih.gov/geo

BioBank Japan Project, http://biobankjp.org

PLINK software, http://pngu.mgh.harvard.edu/~purcell/plink/index.shtml

International HapMap Project, http://www.hapmap.org

EIGENSTRAT software, http://genepath.med.harvard.edu/~reich/Software.htm

MACH and mach2qtl software, http://www.sph.umich.edu/csg/abecasis/MACH/index.html

SNAP, http://www.broadinstitute.org/mpg/snap/index.php

Supporting Information

Figure S1 Principal component analysis (PCA) plot of the subjects. PCA plot of subjects enrolled in the GWAS for SLE. SLE cases and the controls enrolled in the GWAS are plotted based on

eigenvectors 1 and 2 obtained from the PCA using EIGENSTRAT version 2.0 [42], along with European (CEU), African (YRI), Japanese (JPT), and Chinese (CHB) individuals obtained from the Phase II HapMap database (release 22) [29]. Subjects who were estimated to be outliers in terms of ancestry from East-Asian (JPT+CHB) clusters and excluded from the study are indicated by black arrows.
(TIF)

Figure S2 Quantile-Quantile plot (QQ-plot) of *P*-values in the GWAS for SLE. The horizontal axis indicates the expected $-\log_{10}$ (*P*-values). The vertical axis indicates the observed $-\log_{10}$ (*P*-values). The QQ-plot for the *P*-values of all SNPs that passed the quality control criteria is indicated in blue. The QQ-plot for the *P*-values after the removal of SNPs included in the previously reported SLE susceptibility loci is indicated in black. The gray line represents $y = x$. The SNPs for which the *P*-value was smaller than 1.0×10^{-15} are indicated at the upper limit of the plot.
(TIF)

Table S1 Basal characteristics of cohorts.
(DOC)

Table S2 Frequency of clinical characteristics of SLE in this GWAS.
(DOC)

Table S3 Distributions of eQTL positivity rates of the SNPs.
(DOC)

Table S4 Results of replication study 1 for Japanese patients with SLE.
(DOC)

Table S5 Results of replication studies 1 and 2 for Japanese patients with SLE.
(DOC)

Acknowledgments

We are grateful to Dr. A. Miyatake, the members of the Rotary Club of Osaka-Midosuji District 2660 Rotary International in Japan, the autoimmune disease study group, and the staffs of the BioBank Japan Project for supporting the study and clinical sample collection. We thank the members of the Laboratory for Autoimmune Diseases, CGM, RIKEN, for their assistance.

Author Contributions

Conceived and designed the experiments: Y. Okada, K. Shimane, Y. Kochi, A. Suzuki, M. Kubo, N. Kamatani, R. Yamada, Y. Nakamura, K. Yamamoto. Performed the experiments: Y. Kochi, N. Hosono, M. Kubo, K. Myouzen, T. Horiuchi, T. Tahira, K. Hayashi. Analyzed the data: Y. Okada, A. Takahashi, T. Tsunoda, K. Higasa, R. Yamada. Contributed reagents/materials/analysis tools: T. Horita, T. Atsumi, T. Koike, T. Ishii, A. Okamoto, K. Fujio, M. Hirakata, H. Amano, Y. Takasaki, Y. Kondo, S. Ito, T. Sumida, K. Takada, A. Mimori, K. Saito, Y. Tanaka, M. Kamachi, N. Nishimoto, Y. Kawaguchi, C. Terao, K. Ohmura, T. Mimori, F. Matsuda, O.W. Mohammed, K. Matsuda, K. Shimane, K. Ikari, H. Yamanaka. Wrote the paper: Y. Okada, K. Shimane, Y. Kochi, A. Suzuki, R. Yamada, K. Yamamoto.

References

1. Lipsky PE (2001) Systemic lupus erythematosus: an autoimmune disease of B cell hyperactivity. Nat Immunol 2: 764–766.
2. Sestak AL, Shaver TS, Moser KL, Neas BR, Harley JB (1999) Familial aggregation of lupus and autoimmunity in an unusual multiplex pedigree. J Rheumatol 26: 1495–1499.
3. Sigurdsson S, Nordmark G, Goring HH, Lindroos K, Wiman AC, et al. (2005) Polymorphisms in the tyrosine kinase 2 and interferon regulatory factor 5 genes are associated with systemic lupus erythematosus. Am J Hum Genet 76: 528–537.
4. Graham RR, Kozyrev SV, Baechler EC, Reddy MV, Plenge RM, et al. (2006) A common haplotype of interferon regulatory factor 5 (IRF5) regulates splicing and expression and is associated with increased risk of systemic lupus erythematosus. Nat Genet 38: 550–555.
5. Graham RR, Kyogoku C, Sigurdsson S, Vlasova IA, Davies LR, et al. (2007) Three functional variants of IFN regulatory factor 5 (IRF5) define risk and protective haplotypes for human lupus. Proc Natl Acad Sci U S A 104: 6758–6763.
6. Remmers EF, Plenge RM, Lee AT, Graham RR, Hom G, et al. (2007) STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. N Engl J Med 357: 977–986.
7. Cunninghame Graham DS, Graham RR, Manku H, Wong AK, Whittaker JC, et al. (2008) Polymorphism at the TNF superfamily gene TNFSF4 confers susceptibility to systemic lupus erythematosus. Nat Genet 40: 83–89.
8. Nath SK, Han S, Kim-Howard X, Kelly JA, Viswanathan P, et al. (2008) A nonsynonymous functional variant in integrin-alpha(M) (encoded by ITGAM) is associated with systemic lupus erythematosus. Nat Genet 40: 152–154.
9. Harley JB, Alarcon-Riquelme ME, Criswell LA, Jacob CO, Kimberly RP, et al. (2008) Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci. Nat Genet 40: 204–210.
10. Kozyrev SV, Abelson AK, Wojcik J, Zaghlool A, Linga Reddy MV, et al. (2008) Functional variants in the B-cell gene BANK1 are associated with systemic lupus erythematosus. Nat Genet 40: 211–216.
11. Hom G, Graham RR, Modrek B, Taylor KE, Ortmann W, et al. (2008) Association of systemic lupus erythematosus with C8orf13-BLK and ITGAM-ITGAX. N Engl J Med 358: 900–909.
12. Graham RR, Cotsapas C, Davies L, Hackett R, Lessard CJ, et al. (2008) Genetic variants near TNFAIP3 on 6q23 are associated with systemic lupus erythematosus. Nat Genet 40: 1059–1061.
13. Musone SL, Taylor KE, Lu TT, Nititham J, Ferreira RC, et al. (2008) Multiple polymorphisms in the TNFAIP3 region are independently associated with systemic lupus erythematosus. Nat Genet 40: 1062–1064.
14. Han JW, Zheng HF, Cui Y, Sun LD, Ye DQ, et al. (2009) Genome-wide association study in a Chinese Han population identifies nine new susceptibility loci for systemic lupus erythematosus. Nat Genet 41: 1234–1237.
15. Gateva V, Sandling JK, Hom G, Taylor KE, Chung SA, et al. (2009) A large-scale replication study identifies TNIP1, PRDM1, JAZF1, UHRF1BP1 and IL10 as risk loci for systemic lupus erythematosus. Nat Genet 41: 1228–1233.
16. Yang W, Shen N, Ye DQ, Liu Q, Zhang Y, et al. (2010) Genome-wide association study in Asian populations identifies variants in ETS1 and WDFY4 associated with systemic lupus erythematosus. PLoS Genet 6: e1000841. doi:10.1371/journal.pgen.1000841.
17. Lessard CJ, Adrianto I, Kelly JA, Kaufman KM, Grundahl KM, et al. (2011) Identification of a systemic lupus erythematosus susceptibility locus at 11p13 between PDHX and CD44 in a multiethnic study. Am J Hum Genet 88: 83–91.
18. Yang J, Yang W, Hirankarn N, Ye DQ, Zhang Y, et al. (2011) ELF1 is associated with systemic lupus erythematosus in Asian populations. Hum Mol Genet 20: 601–607.
19. Hopkinson ND, Doherty M, Powell RJ (1994) Clinical features and race-specific incidence/prevalence rates of systemic lupus erythematosus in a geographically complete cohort of patients. Ann Rheum Dis 53: 675–680.
20. Danchenko N, Satia JA, Anthony MS (2006) Epidemiology of systemic lupus erythematosus: a comparison of worldwide disease burden. Lupus 15: 308–318.
21. Yang J, Benyamin B, McEvoy BP, Gordon S, Henders AK, et al. (2010) Common SNPs explain a large proportion of the heritability for human height. Nat Genet 42: 565–569.
22. Raychaudhuri S, Plenge RM, Rossin EJ, Ng AC, Purcell SM, et al. (2009) Identifying relationships among genomic disease regions: predicting genes at pathogenic SNP associations and rare deletions. PLoS Genet 5: e1000534. doi:10.1371/journal.pgen.1000534.
23. Cantor RM, Lange K, Sinsheimer JS (2010) Prioritizing GWAS results: A review of statistical methods and recommendations for their application. Am J Hum Genet 86: 6–22.
24. Dubois PC, Trynka G, Franke L, Hunt KA, Romanos J, et al. (2010) Multiple common variants for celiac disease influencing immune gene expression. Nat Genet 42: 295–302.
25. Cookson W, Liang L, Abecasis G, Moffatt M, Lathrop M (2009) Mapping complex disease traits with global gene expression. Nat Rev Genet 10: 184–194.
26. Kochi Y, Okada Y, Suzuki A, Ikari K, Terao C, et al. (2010) A regulatory variant in CCR6 is associated with rheumatoid arthritis susceptibility. Nat Genet 42: 515–519.
27. Yamaguchi-Kabata Y, Nakazono K, Takahashi A, Saito S, Hosono N, et al. (2008) Japanese population structure, based on SNP genotypes from 7003 individuals compared to other ethnic groups: effects on population-based association studies. Am J Hum Genet 83: 445–456.
28. Stranger BE, Nica AC, Forrest MS, Dimas A, Bird CP, et al. (2007) Population genomics of human gene expression. Nat Genet 39: 1217–1224.
29. The International HapMap Consortium (2003) The International HapMap Project. Nature 426: 789–796.

30. Pearson JV, Huentelman MJ, Halperin RF, Tembe WD, Melquist S, et al. (2007) Identification of the genetic basis for complex disorders by use of pooling-based genomewide single-nucleotide-polymorphism association studies. Am J Hum Genet 80: 126–139.
31. Xia ZB, Popovic R, Chen J, Theisler C, Stuart T, et al. (2005) The MLL fusion gene, MLL-AF4, regulates cyclin-dependent kinase inhibitor CDKN1B (p27kip1) expression. Proc Natl Acad Sci U S A 102: 14028–14033.
32. Isnard P, Core N, Naquet P, Djabali M (2000) Altered lymphoid development in mice deficient for the mAF4 proto-oncogene. Blood 96: 705–710.
33. Schadt EE, Molony C, Chudin E, Hao K, Yang X, et al. (2008) Mapping the genetic architecture of gene expression in human liver. PLoS Biol 6: e107. doi:10.1371/journal.pbio.0060107.
34. Ernst J, Kheradpour P, Mikkelsen TS, Shoresh N, Ward LD, et al. (2011) Mapping and analysis of chromatin state dynamics in nine human cell types. Nature 473: 43–49.
35. Stahl EA, Raychaudhuri S, Remmers EF, Xie G, Eyre S, et al. (2010) Genome-wide association study meta-analysis identifies seven new rheumatoid arthritis risk loci. Nat Genet 42: 508–514.
36. Nakamura Y (2007) The BioBank Japan Project. Clin Adv Hematol Oncol 5: 696–697.
37. Hochberg MC (1997) Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 40: 1725.
38. Suzuki A, Yamada R, Kochi Y, Sawada T, Okada Y, et al. (2008) Functional SNPs in CD244 increase the risk of rheumatoid arthritis in a Japanese population. Nat Genet 40: 1224–1229.
39. Shimane K, Kochi Y, Horita T, Ikari K, Amano H, et al. (2010) The association of a nonsynonymous single-nucleotide polymorphism in TNFAIP3 with systemic lupus erythematosus and rheumatoid arthritis in the Japanese population. Arthritis Rheum 62: 574–579.
40. Myouzen K, Kochi Y, Shimane K, Fujio K, Okamura T, et al. (2010) Regulatory polymorphisms in EGR2 are associated with susceptibility to systemic lupus erythematosus. Hum Mol Genet 19: 2313–2320.
41. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, et al. (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet 81: 559–575.
42. Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, et al. (2006) Principal components analysis corrects for stratification in genome-wide association studies. Nat Genet 38: 904–909.
43. Li Y, Willer C, Sanna S, Abecasis G (2009) Genotype imputation. Annu Rev Genomics Hum Genet 10: 387–406.
44. Okada Y, Takahashi A, Ohmiya H, Kumasaka N, Kamatani Y, et al. (2011) Genome-wide association study for C-reactive protein levels identified pleiotropic associations in the IL6 locus. Hum Mol Genet 20: 1224–1231.
45. Aikawa Y, Yamamoto M, Yamamoto T, Morimoto K, Tanaka K (2002) An anti-rheumatic agent T-614 inhibits NF-kappaB activation in LPS- and TNF-alpha-stimulated THP-1 cells without interfering with IkappaBalpha degradation. Inflamm Res 51: 188–194.
46. Akamatsu S, Takata R, Ashikawa K, Hosono N, Kamatani N, et al. (2010) A functional variant in NKX3.1 associated with prostate cancer susceptibility down-regulates NKX3.1 expression. Hum Mol Genet 19: 4265–4272.
47. Johnson AD, Handsaker RE, Pulit SL, Nizzari MM, O'Donnell CJ, et al. (2008) SNAP: a web-based tool for identification and annotation of proxy SNPs using HapMap. Bioinformatics 24: 2938–2939.

呼吸器内科，26(3)：220-223，2014

Lecture

解　説

膠原病における疾患関連マーカーと肺病変*

川　口　鎮　司**

Key Words：interstitial lung disease, systemic sclerosis, idiopathic inflammatory myopathy, rheumatoid arthritis(RA)

はじめに

膠原病に間質性肺炎を合併することはよく知られている．近年，特発性間質性肺炎と診断された症例の中に膠原病にみられる自己抗体を有している症例が報告された[1]．それらの症例では，間質性肺炎に副腎皮質ステロイド薬が有効であることが多い．自己抗体が陽性であっても膠原病と診断できるかどうかは臨床症状や他の検査結果によるところが大きい．間質性肺炎を合併する膠原病の疾患関連マーカーに関して解説する．

間質性肺病変を合併する膠原病は，関節リウマチ，シェーグレン症候群，全身性強皮症，多発性筋炎，皮膚筋炎，血管炎である．全身性エリテマトーデスにおいても稀に間質性肺病変を合併するとされるが疾患活動性として問題になることはほとんどない．上記の間質性肺病変をしばしば合併する疾患に関して，その臨床症状と疾患関連マーカーである自己抗体に関して各疾患ずつにわけて述べる．

関節リウマチ(rheumatoid arthritis；RA)

関節リウマチは，手指や足趾などの小関節を中心に多発性の関節炎を主症状とする．診断に用いられる疾患関連マーカーは，リウマトイド因子(RF)と抗CCP抗体である[2]．ともに感度，特異度は，80%以上と種々の報告があり有用性は高い．リウマチ肺と呼ばれる関節リウマチに合併する間質性肺炎は，HRCT上，蜂巣肺を呈するUIP(usual interstitial pneumonia)パターンを呈することが多く，その進行は非常に緩徐である．合併する頻度は，報告により違いがあるが，19～33%とされている[3]．間質性肺炎を合併する症例では，RFが著明に高値であり，自己免疫の関与が示唆される．慢性的な経過をとる症例が多いが，肺炎などの感染症を契機に急性増悪することもあり，定期的なHRCTでの検査が必要である．また，器質化肺炎(organizing pneumonia；OP)を合併する．免疫抑制療法を行っているため，感染性肺炎が問題となることが多いが，浸潤影を呈し，抗菌薬治療が無効な病態にOPがある．OPの治療は副腎皮質ステロイド薬であり，感染性肺炎とは大きく異なるので，それらの鑑別は重要である[4]．

シェーグレン症候群(Sjogren's syndrome)

関節リウマチに次いで患者数が多い膠原病は，シェーグレン症候群であると推定される．男女比が1：14程度で圧倒的に女性に多い疾患である．シェーグレン症候群の疾患関連マーカーは，抗SSA(Ro)抗体と抗SSB(La)抗体である．特徴的な血液検査所見としては，高ガンマグロブリン血症，抗核抗体陽性，リウマトイド因子陽性がしばしば認められる．一般的ではないが，抗フォドリン抗体[5]，抗ムスカリン受容体抗体[6]もシェーグレン症候群に特異的な自己抗体として報告さ

* Lung disease associated with connective tissue diseases.

** Yasushi KAWAGUCHI, M.D., Ph.D.: 東京女子医科大学リウマチ科〔〒162-0054 東京都新宿区河田町10-22〕; Institute of Rheumatology, Tokyo Women's Medical University, Tokyo 162-0054, JAPAN

表1 全身性強皮症と疾患関連マーカー

自己抗体	臨床症状
抗 topoisomerase I 抗体 (抗Scl-70抗体)	びまん性皮膚硬化，間質性肺病変
抗RNA polymerase I/III 抗体	びまん性皮膚硬化，強皮症腎
抗U3 RNP抗体	びまん性皮膚硬化，筋炎，下部腸管病変
抗U1 RNP抗体	関節炎，発熱，白血球減少症，肺高血圧症 皮膚硬化はびまん性と限局性がほぼ同頻度
抗セントロメア抗体	限局性皮膚硬化，CREST症候群，肺高血圧症
抗Th/To抗体	限局性皮膚硬化，間質性肺病変
抗Ku抗体	限局性皮膚硬化，筋炎

れている．臨床症状としては，乾燥症状が共通してみられる所見である．特に涙と唾液の減少が特徴的である．これらは，自己反応性のリンパ球が，涙腺あるいは唾液腺において炎症をひき起こしていると考えられている．外分泌腺を対象として自己免疫異常を呈する疾患であるが，外分泌腺以外にも病変がみられる．間質性肺炎の合併は，22％にみられる．HRCT画像では，NSIP(non-specific interstitial pneumonia)の頻度が最も高く，LIP(lymphoid interstitial pneumonia)の頻度は少ない．

全身性強皮症 (systemic sclerosis；SSc)

日本では，2万人程度の患者がいると推定されている．男女比は1：8～10と女性に多い．レイノー現象などの血管障害が初発症状であり，徐々に皮膚が硬くなることに気づく．多臓器の線維化が主症状である．皮膚の線維化だけでなく，肺の線維化が生じ，間質性肺病変の合併が生命予後に関与する．60～70％の症例に間質性肺疾患を合併し，免疫抑制療法の適応となることが多い[7]．また，全身性強皮症は，90％以上の症例で抗核抗体が陽性となり，その多くの対応抗原が同定されている．それらの自己抗体の種類を表1に示す．これらの自己抗体は，全身性強皮症に特異性が高い．また，それぞれの自己抗体は，全身性強皮症の臨床症状とよく関連する．抗U1-RNP抗体は，全身性エリテマトーデスや多発性筋炎の合併がみられる．また，抗Ku抗体は，多発性筋炎との重複症候群に認められる．

間質性肺病変は，抗Scl-70抗体や抗Th/To抗体陽性の全身性強皮症に高頻度である．間質性肺疾患は線維化の病態であり，皮膚硬化が重症であるびまん性皮膚硬化型に合併することが多いとされる．一方，抗Th/To抗体の症例では，限局皮膚硬化型であるが，間質性肺病変を高頻度に(60％以上)合併する．抗RNAポリメラーゼIII抗体陽性の全身性強皮症では，皮膚硬化はびまん性に広がり，難治性で高度の皮膚硬化を呈するが，間質性肺病変は軽度であることが多い[8]．これらのことから，肺の線維化は，必ずしも，皮膚硬化の重症度と相関しないことがわかってきた．

一方，血管病変において最も生命予後にかかわるのは，肺動脈性肺高血圧症である[9]．全身性強皮症では，8～10％に合併する．抗セントロメア抗体，抗U1-RNP抗体陽性の症例で頻度が高いことが知られている．これらの自己抗体を有している症例では，肺動脈性肺高血圧症の合併に留意して，心臓超音波検査を定期的に行う必要がある．

多発性筋炎(polymyositis；PM)，皮膚筋炎(dermatomyositis；DM)

PM，DMは，本邦では，1万7千人程度と推定されている．男女比は，1：3～4と女性に多い．PMは，皮膚症状がなく，近位筋に炎症が認められ，筋力の低下が生じる．骨格筋の病理検査からは，非壊死性筋線維に浸潤するT細胞はCD8陽性細胞で，筋内に遊走している．これらのT細胞は自己の筋細胞に対してHLA class I 抗原拘束性の細胞傷害能を有する．これらの浸潤しているT細胞のT細胞受容体は単一であることから，なんらかの自己抗原に反応して集積していることが推測される．一方，DMは，特徴的な皮膚症状を呈する筋炎疾患である．皮膚症状としては，

表2　多発性筋炎，皮膚筋炎と疾患関連マーカー

自己抗体	臨床症状
抗アミノアシルtRNA合成酵素抗体	間質性肺炎(亜急性あるいは慢性の進行)
抗Jo-1抗体	
抗PL-7抗体	
抗PL-12抗体	
抗OJ抗体	
抗EJ抗体	
抗KS抗体	
抗MDA5抗体	筋症状が軽度の皮膚筋炎，急性間質性肺炎
抗SRP抗体	ステロイド抵抗性の筋症
抗Mi-2抗体	皮膚筋炎，治療反応性良好
抗TIF-1γ抗体	悪性腫瘍を合併する皮膚筋炎

ヘリオトロープ疹とゴットロン徴候が特徴的である．DMの筋炎所見では，主にB細胞とCD4陽性のT細胞浸潤が有意で，血管周囲にみられる．これらの病理所見からは，PMとDMの筋炎所見では異なった病態を呈していることがわかる．これらのPM，DMに対して疾患関連マーカーとして種々の自己抗体がある(表2)．他の膠原病と異なりこれらの多くは，抗核抗体ではなく，抗細胞質抗体である[10]．

近年，amyopathic DM(ADM)あるいはclinically amyopathic DM(CADM)という，筋炎症状がないが特徴的なDMの皮膚症状を呈する疾患があることがわかってきた[11]．このADM/CADMの特徴は，急速に進行する間質性肺炎を高頻度に合併することである．表2に示したように，抗MDA5(melanoma differentiation-associated gene 5)抗体は，ADM/CADM症例に特異的にみられる自己抗体である[12]．抗MDA5抗体陽性のADM/CADM症例においては，80%以上に急性間質性肺炎(acute interstitial pneumonia；AIP)がみられる．このAIPの重症度とは，血清フェリチン値がよく相関することがわかってきた．ADM/CADMに合併したAIPが進行すると免疫抑制療法に抵抗性で致命率が高いとことがわかっていた．そのため，現在では，HRCT所見での肺病変が軽度でも血清フェリチン値が，500 ng/ml以上であれば，早期から強力な免疫抑制療法を行うことが推奨される[13]．

PM，DMの抗アミノアシルtRNA合成酵素(aminoacyl tRNA synthetase；ARS)抗体は，現在，6種類以上の種類が報告されている．これらのなかで，最も頻度が高いものが，抗Jo-1抗体である．これらの抗ARS抗体は，亜急性あるいは慢性に進行する間質性肺炎を高頻度(80%以上)に合併する．しかし，これらの間質性肺炎は，副腎皮質ステロイド薬などの免疫抑制療法が有効であり，生命予後は比較的良好である．この抗ARS抗体を有する疾患群は，筋炎，関節炎と間質性肺炎を同時に合併することが特徴である．特に，抗PL-12抗体陽性では筋炎症状に乏しく間質性肺炎だけがみられ，特発性間質性肺炎と診断されている症例に認められることがある．

血管炎

血管炎の疾患関連マーカーは，抗好中球細胞質抗体(anti-neutrophil cytoplasmic antibody；ANCA)が知られている．ANCAには，2種類の対応抗原があり，それぞれ，MPO(myeloperoxidase)-ANCAとPR3(proteinase 3)-ANCAとされている．これらのANCAが認められる血管炎をANCA関連血管炎とよぶ[14]．これらの血管炎には，その臨床症状により，顕微鏡的多発血管炎(microscopic polyangiitis；MPA)，多発血管炎性肉芽腫症(granulomatosis with polyangiitis；GPA)，好酸球性多発血管炎性肉芽腫症(eosinophilic granulomatosis with polyangiitis；EGPA)がある．GPA，EGPAは，以前は，Wegener肉芽腫症，Churg-Strauss症候群と呼ばれていた．MPAには，高頻度に間質性肺炎および肺胞出血が高頻度に合併する．また，GPA，EGPAには，肺に肉芽腫性血管炎病変を合併する．

文　献

1) Gutsche M, Rosen GD, Swigris JJ. Connective tissue disease-associated interstitial lung disease : A review. Curr Respir Care Rep 2012 ; 1 : 224.
2) Shidara K, Inoue E, Tanaka E, et al. Comparison of the second and third generation anti-cyclic citrullinated peptide antibody assays in the diagnosis of Japanese patients with rheumatoid arthritis. Rheumatol Int 2011 ; 31 : 617.
3) Kim EJ, Elicker BM, Maldonado F, et al. Usual interstitial pneumonia in rheumatoid arthritis-associated interstitial lung disease. Eur Respir J 2010 ; 35 : 1322.
4) 徳田　均．膠原病の間質性肺疾患up to date膠原病におけるCOPの診断と治療．リウマチ科 2009；42：175.
5) Haneji N, Nakamura T, Takio K, et al. Identification of α-fodrin as a candidate autoantigen in primary Sjogren's syndrome. Science 1997 ; 276 : 604.
6) Naito Y, Matsumoto I, Wakamatsu E, et al. Altered peptide ligands regulate muscarinic acetylcholine receptor reactive T cells of patients with Sjogren's syndrome. Ann Rheum Dis 2006 ; 65 : 269.
7) Tochimoto A, Kawaguchi Y, Hara M, et al. Efficacy and safety of intravenous cyclophosphamide pulse therapy with oral prednisolone in the treatment of interstitial lung disease with systemic sclerosis : four-year follow-up. Modern Rheumatol 2011 ; 21 : 296.
8) Okano Y, Steen VD, Medsger TA Jr. Autoantibody reactive with RNA polymerase III in systemic sclerosis. Ann Intern Med 1993 ; 119 : 1005.
9) 川口鎮司．膠原病性肺高血圧症治療の新たな展開．Ther Res 2010；31：1189.
10) Targoff IN. Myositis specific autoantibodies. Curr Rheumatol Rep 2006 ; 8 : 196.
11) Gerami P, Schope JM, McDonald L, et al. A systematic review of adult-onset clinically amyopathic dermatomyositis : a missing link within the spectrum of the idiopathic inflammatory myopathies. J Am Acad Dermatol 2006 ; 54 : 597.
12) Sato S, Hirakata M, Kuwana M, et al. Autoantibodies to a 140-kd polypeptide, CADM-140, in Japanese patients with clinically amyopathic dermatomyositis. Arthritis Rheum 2005 ; 52 : 1571.
13) Gono T, Kawaguchi Y, Hara M, et al. Increased ferritin predicts development and severity of acute interstitial lung disease as a complication of dermatomyositis. Rheumatology 2010 ; 49 : 1354.
14) Jannette JC, Falk RJ, Bacon PA, et al. 2012 revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides. Arthritis Rheum 2013 ; 65 : 1.

*　　*　　*