PLOS ONE

RESEARCH ARTICLE

Human Leukocyte Antigen and Systemic Sclerosis in Japanese: The Sign of the Four Independent Protective Alleles, *DRB1*13:02*, *DRB1*14:06*, *DQB1*03:01*, and *DPB1*02:01*

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Citation: Furukawa H, Oka S, Kawasaki A, Shimada K, Sugii S, Matsushita T, et al. (2016) Human Leukocyte Antigen and Systemic Sclerosis in Japanese: The Sign of the Four Independent Protective Alleles, *DRB1*13:02*, *DRB1*14:06*, *DQB1*03:01*, and *DPB1*02:01*. PLoS ONE 11(4): e0154255. doi:10.1371/journal.pone.0154255

Editor: Shervin Assassi, University of Texas Health Science Center at Houston, UNITED STATES

Received: December 27, 2015

Accepted: April 11, 2016

Published: April 26, 2016

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The study was supported by Grants-in-Aid for Scientific Research (B, C) (26293123, 22591090) and for Young Scientists (B) (24791018) from the Japan Society for the Promotion of Science, Health and Labour Science Research Grants from the Ministry of Health, Labour, and Welfare of Japan, Grants-in-Aid for Clinical Research from National Hospital Organization, Research Grants from Daiwa Securities Health Foundation, Research Grants from

Abstract

Objective

Several studies on associations between human leukocyte antigen (HLA) allele frequencies and susceptibility to systemic sclerosis (SSc) have been reported. Anti-centromere antibodies (ACA) and anti-topoisomerase I antibodies (ATA) are found in SSc patients. Here, we

Japan Research Foundation for Clinical Pharmacology, Research Grants from The Nakatomi Foundation, Research Grants from Takeda Science Foundation, Research Grants from Mitsui Sumitomo Insurance Welfare Foundation, Research Grants from Kato Memorial Trust for Nambyo Research, and research grants from the following pharmaceutical companies: Abbott Japan Co., Ltd., Astellas Pharma Inc., Chugai Pharmaceutical Co., Ltd., Eisai Co., Ltd., Mitsuibishi Tanabe Pharma Corporation, Merck Sharp and Dohme Inc., Pfizer Japan Inc., Takeda Pharmaceutical Company Limited, Teijin Pharma Limited. The funders had no role in study design, data collection and analysis, decision to publish, or preparing the manuscript.

Competing Interests: HF has the following conflicts, and the following funders are supported wholly or in part by the indicated pharmaceutical companies. The Japan Research Foundation for Clinical Pharmacology is run by Daiichi Sankyo, the Takeda Science Foundation is supported by an endowment from Takeda Pharmaceutical Company and the Nakatomi Foundation was established by Hisamitsu Pharmaceutical Co., Inc. The Daiwa Securities Health Foundation was established by Daiwa Securities Group Inc. and Mitsui Sumitomo Insurance Welfare Foundation was established by Mitsui Sumitomo Insurance Co., Ltd. HF received honoraria from Ajinomoto Co., Inc., Daiichi Sankyo Co., Ltd., Dainippon Sumitomo Pharma Co., Ltd., Pfizer Japan Inc., and Takeda Pharmaceutical Company. ST was supported by research grants from 9 pharmaceutical companies: Abbott Japan Co., Ltd., Astellas Pharma Inc., Chugai Pharmaceutical Co., Ltd., Eisai Co., Ltd., Mitsubishi Tanabe Pharma Corporation, Merck Sharp and Dohme Inc., Pfizer Japan Inc., Takeda Pharmaceutical Company Limited, Teijin Pharma Limited. ST received honoraria from Asahi Kasei Pharma Corporation, Astellas Pharma Inc., AbbVie GK., Chugai Pharmaceutical Co., Ltd., Ono Pharmaceutical Co., Ltd., Mitsubishi Tanabe Pharma Corporation, Pfizer Japan Inc. The other authors declare no financial or commercial conflict of interest. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

sought to identify *HLA* alleles associated with SSc in Japanese, and explored their associations with SSc phenotypes including the presence of autoantibodies.

Methods

Associations of *HLA-DRB1*, *DQB1*, *and DPB1* were analyzed in 463 Japanese SSc patients and 413 controls.

Results

We found that *DRB1*13:02* ($P = 0.0011$, $Pc = 0.0319$, odds ratio [OR] 0.46, 95% confidence interval [CI] 0.29–0.73), *DRB1*14:06* ($P = 6.60X10^{-5}$, $Pc = 0.0020$, OR 0.05, 95%CI 0.01–0.41), *DQB1*03:01* ($P = 0.0009$, $Pc = 0.0150$, OR 0.56, 95%CI 0.40–0.79), and *DPB1*02:01* ($P = 5.16X10^{-6}$, $Pc = 8.77X10^{-5}$, OR 0.52, 95%CI 0.39–0.69) were protectively associated with SSc. In addition, these four alleles seemed to be independently associated with the protection against the susceptibility of SSc. On the other hand, we could not find predisposing alleles for overall SSc. With respect to SSc subsets, a tendency for these four alleles to be protectively associated was observed. However, there was a significant association between *DRB1*01:01*, *DRB1*10:01*, *DQB1*05:01*, and *DPB1*04:02* and the susceptibility to SSc with ACA. On the other hand, the presence of *DRB1*15:02*, *DQB1*06:01*, *DPB1*03:01*, and *DPB1*09:01* was associated with SSc with ATA.

Conclusion

Thus, the present study has identified protective associations of the four *HLA* class II alleles with overall Japanese SSc and predisposing associations of *HLA* class II alleles with Japanese SSc subsets.

Introduction

Systemic sclerosis (SSc) is a complex autoimmune disease of unknown etiology that is characterized by fibrosis of the skin and internal organs, small-vessel vasculopathy, and the production of anti-nuclear antibodies. It is a chronic autoimmune disease, susceptibility to which is associated with genetic and environmental factors [1,2]. Genetic risk factors for SSc include alleles of the loci *HLA-DRB1*, *DQB1*, *DPB1*, *DPB2*, *IRF5*, *STAT4*, *CD247*, *IRF4*, and others [1,3,4,5]. Thus, a functional role of these polymorphisms in SSc has been suggested, as well as relationships with other autoimmune diseases such as rheumatoid arthritis or systemic lupus erythematosus. In particular, skewed frequencies of human leukocyte antigen (*HLA*) alleles are known to be associated with SSc. Different *HLA* class II alleles appear to be associated with SSc susceptibility in different ethnic groups, such as *HLA-DRB1*11:04*, *DQB1*03:01*, or *DQB1*26 epi* (*DQB1* alleles encoding a non-leucine residue at position 26) in Europeans[2,6,7], *DRB1*08:04*, *DQB1*03:01*, or *DPB1*13:01* in African-Americans [6], and *DRB1*15:02*, *DQB1*05:01*, and *DPB1*03:01* in Asians [8,9,10,11,12,13,14].

SSc presents with several specific autoantibodies including anti-centromere antibodies (ACA) [15] and anti-topoisomerase I antibodies (ATA, also termed Scl-70) [16]. ACA are present in a subset of patients with SSc who have limited cutaneous SSc (lcSSc). This is characterized by skin thickening that is relatively restricted to the fingers and hands, with less severe

internal organ involvement. ATA are present in an SSc subset having diffuse cutaneous SSc (dcSSc), in which skin lesions are extensive and progressive, and serious internal organ involvement is observed. *DQB1*05:01*, *DQB1*26 epi* are associated with SSc with ACA in people of European descent [6] and *DQB1*05:01* in Japanese [9]. Several studies have also shown that certain *DRB1* or *DPB1* alleles are associated with SSc with ATA; thus, *DPB1*13:01* is associated with SSc with ATA in Europeans [6] and *DRB1*15:02* and *DPB1*09:01* in Japanese [9].

Here, we sought *HLA* alleles predisposing and protective to SSc in Japanese, and explored their associations with SSc phenotypes including the presence of autoantibodies.

Materials and Methods

Patients and healthy controls

SSc patients were recruited at Sagamihara Hospital, Yokohama Minami Kyosai Hospital, Tama Medical Center, Kitasato University, Komagome Hospital, Teikyo University, Himeji Medical Center, Morioka Hospital, Kyushu Medical Center, Nagoya Medical Center, Nagasaki Medical Center, University of Tsukuba, and Kanazawa University. Healthy controls (n = 413; mean age ± SD, 41.4 ± 12.6 years, 62 male [14.0%]) were recruited without matching at Sagamihara Hospital, Teikyo University, and Kanazawa University or by the Pharma SNP Consortium (Tokyo, Japan) [17]. All patients and healthy individuals were native Japanese living in Japan. All patients with SSc fulfilled the American College of Rheumatology criteria for SSc [18] and were classified as dcSSc and lcSSc according to the classification criteria by LeRoy et al. [19]. ACA was detected using Mesacup-2 test CENP-B enzyme-linked immunosorbent assay (ELISA, Medical & Biological Laboratories, Nagoya, Japan). ATA was detected using Mesacup-3 test Scl-70 ELISA (Medical & Biological Laboratories) or Ouchterlony double immunodiffusion method (TFB, Hachioji, Japan). This study was reviewed and approved by the research ethics committees of each participating institute as follows: Sagamihara Hospital Research Ethics Committee, Yokohama Minami Kyosai Hospital Research Ethics Committee, Tama Medical Center Research Ethics Committee, Kitasato University Research Ethics Committee, Komagome Hospital Research Ethics Committee, Teikyo University Research Ethics Committee, Himeji Medical Center Research Ethics Committee, Morioka Hospital Research Ethics Committee, Kyushu Medical Center Research Ethics Committee, Nagoya Medical Center Research Ethics Committee, Nagasaki Medical Center Research Ethics Committee, Kanazawa University Research Ethics Committee, and University of Tsukuba Research Ethics Committee. Written informed consent was obtained from all study participants. This study was conducted in accordance with the principles expressed in the Declaration of Helsinki.

Genotyping

Genotyping of *HLA-DRB1*, *DQB1*, and *DPB1* was performed by the polymerase chain reaction technique with sequence-specific oligonucleotide probes (WAKFlow HLA typing kits, Wakunaga, Hiroshima, Japan), using the Bio-Plex 200 system (Bio-Rad, Hercules, CA). *DQB1*26 epi* alleles are *DQB1*03:01*, **04:01*, **04:02*, **05:01*, **05:02*, **05:03*, and **06:01*. Results of genotyping for some of the healthy controls were reported previously [20,21,22].

Statistical analysis

Differences of SSc characteristics were analyzed by Student's t-test or Fisher's exact test using 2X2 contingency tables. The Hardy-Weinberg exact tests were performed by the Markov chain method under the condition of 10000 each of dememorization, batches, and iterations per batch (Genepop on the web; http://genepop.curtin.edu.au/) [23]. The statistical power in each

condition of allele carrier frequency and odds ratio was calculated on the sample size of this study (463 overall SSc patients and 413 controls, S1 Fig) by PS: Power and Sample Size Calculation version 3.1.2 (http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize) [24]. Differences of allele carrier frequencies were analyzed by Fisher's exact test using 2X2 contingency tables under the dominant model. Adjustment for multiple comparisons was performed using the Bonferroni method. Corrected *P* (*Pc*) values were calculated by multiplying the *P* value by the number of alleles tested. Relative predispositional effects (RPE) were analyzed by sequential elimination of carriers of each allele with the strongest association [25]. To examine whether each protective *HLA* class II allele independently contributes to the protection of SSc, multiple logistic regression analysis under the additive model was employed and the deviation from 0 was evaluated for coefficients by the Wald test. *DRB1-DQB1-DPB1* haplotype frequencies were estimated using the expectation-maximization method by SNPAlyze ver.8.0.4 Pro software (Dynacom, Chiba, Japan). *P* values were calculated by permutation test (100000 permutations). Differences of amino acid residue carrier frequencies (amino acid residue carrier vs. non-carrier) were analyzed by Fisher's exact test using 2X2 contingency tables under the dominant model on the detected polymorphic amino acid positions in the β1 domain of HLA-DRβ, DQβ, and DPβ chains. Adjustment for multiple comparisons was performed using the Bonferroni method. *Pc* values were calculated by multiplying the *P* value by the number of amino acid positions tested.

Results

Clinical features of the SSc patients

Characteristics of the SSc patients are described in Table 1. Mean age and ACA positivity in dcSSc were lower than in lcSSc. ATA positivity and percentage of male were higher in dcSSc than lcSSc.

HLA association analysis of SSc patients

HLA-DRB1, *DQB1*, *and DPB1* genotyping was performed in SSc patients and healthy controls to compare carrier frequencies of each allele. No deviation from Hardy-Weinberg equilibrium was detected in the controls (*DRB1*: $P = 0.4327$, *DQB1*: $P = 0.2136$, *DPB1*: $P = 0.7464$, all locus: $P = 0.5000$), though a deviation was observed in the overall SSc patients (*DRB1*: $P = 0.1017$, *DQB1*: $P = 0.0769$, *DPB1*: $P = 0.0260$, all locus: $P = 0.0093$). A significant protective association was found for *DRB1*13:02* ($P = 0.0011$, $Pc = 0.0319$, odds ratio [OR] 0.46, 95% confidence interval [CI] 0.29–0.73, Table 2) and *DRB1*14:06* ($P = 6.60\text{X}10^{-5}$, $Pc = 0.0020$, OR 0.05, 95%CI 0.01–0.41, Table 2) with SSc. A significant association with resistance to SSc was also found for

Table 1. Characteristics of the SSc patients.

	SSc	dcSSc	lcSSc	*P*
Number	463	157	266	
Mean age, years (SD)	58.4 (13.5)	54.5 (14.9)	61.0 (12.0)	$*4.65\text{X}10^{-6}$
Male, n (%)	50 (10.9)	29 (18.5)	19 (7.2)	0.0007
ACA positive, n (%)	194 (44.9)	20 (12.9)	167 (65.0)	$1.73\text{X}10^{-26}$
ATA positive, n (%)	119 (27.4)	85 (54.5)	27 (10.5)	$3.17\text{X}10^{-22}$

SSc: systemic sclerosis, dcSSc: diffuse cutaneous SSc, lcSSc: limited cutaneous SSc, ACA: anti-centromere antibodies, ATA: anti-topoisomerase I antibodies. Association was tested between dcSSc and lcSSc by Fisher's exact test using 2X2 contingency tables or Student's t-test.

* Student's t-test was employed.

doi:10.1371/journal.pone.0154255.t001

Table 2. *HLA-DRB1* allele carrier frequencies in the SSc patients and the healthy controls.

	Case (n = 463)	Control (n = 413)	*P*	OR	*Pc*	95%CI	*P* (RPE)
*DRB1*01:01*	65 (14.0)	42 (10.2)	0.0979	1.44	NS		
*DRB1*03:01*	1 (0.2)	2 (0.5)	0.6044	0.44	NS		
*DRB1*04:01*	10 (2.2)	7 (1.7)	0.8071	1.28	NS		
*DRB1*04:03*	39 (8.4)	19 (4.6)	0.0288	1.91	0.8633	(1.08–3.36)	0.0343
*DRB1*04:04*	6 (1.3)	0 (0.0)	0.0322	11.75	0.9672	(0.66–209.22)	
*DRB1*04:05*	94 (20.3)	87 (21.1)	0.8023	0.95	NS		
*DRB1*04:06*	31 (6.7)	34 (8.2)	0.4390	0.80	NS		
*DRB1*04:07*	11 (2.4)	3 (0.7)	0.0610	3.33	NS		0.0439
*DRB1*04:10*	23 (5.0)	14 (3.4)	0.3129	1.49	NS		
*DRB1*07:01*	7 (1.5)	3 (0.7)	0.3490	2.10	NS		
*DRB1*08:02*	56 (12.1)	38 (9.2)	0.1896	1.36	NS		
*DRB1*08:03*	79 (17.1)	61 (14.8)	0.4059	1.19	NS		
*DRB1*08:09*	0 (0.0)	1 (0.2)	0.4715	0.30	NS		
*DRB1*09:01*	116 (25.1)	105 (25.4)	0.9379	0.98	NS		
*DRB1*10:01*	15 (3.2)	2 (0.5)	0.0027	6.88	0.0820	(1.56–30.27)	0.0059
*DRB1*11:01*	17 (3.7)	22 (5.3)	0.2540	0.68	NS		
*DRB1*12:01*	26 (5.6)	29 (7.0)	0.4058	0.79	NS		
*DRB1*12:02*	7 (1.5)	10 (2.4)	0.3407	0.62	NS		
*DRB1*13:01*	4 (0.9)	5 (1.2)	0.7419	0.71	NS		
*DRB1*13:02*	32 (6.9)	57 (13.8)	0.0011	0.46	0.0319	(0.29–0.73)	0.0007
*DRB1*14:03*	15 (3.2)	21 (5.1)	0.1772	0.63	NS		
*DRB1*14:04*	0 (0.0)	1 (0.2)	0.4715	0.30	NS		
*DRB1*14:05*	21 (4.5)	14 (3.4)	0.4900	1.35	NS		
*DRB1*14:06*	1 (0.2)	16 (3.9)	$6.60X10^{-5}$	0.05	0.0020	(0.01–0.41)	$6.60X10^{-5}$
*DRB1*14:07*	0 (0.0)	1 (0.2)	0.4715	0.30	NS		
*DRB1*14:29*	1 (0.2)	0 (0.0)	1.0000	2.68	NS		
*DRB1*14:54*	23 (5.0)	28 (6.8)	0.3117	0.72	NS		
*DRB1*15:01*	55 (11.9)	68 (16.5)	0.0521	0.68	NS		
*DRB1*15:02*	120 (25.9)	89 (21.5)	0.1323	1.27	NS		
*DRB1*16:02*	5 (1.1)	5 (1.2)	1.0000	0.89	NS		
DR6	91 (19.7)	137 (33.2)	$7.08X10^{-6}$	0.49		(0.36–0.67)	

SSc: systemic sclerosis, OR: odds ratio, CI: confidence interval, *Pc*: corrected *P* value, NS: not significant, RPE: relative predispositional effects. Allele carrier frequencies are shown in parenthesis (%). Association was tested by Fisher's exact test using 2X2 contingency tables under the dominant model. RPE were tested by sequential elimination of carriers of each of the alleles *DRB1*14:06*, **13:02*, **10:01*, **04:03*, and **04:07*.

doi:10.1371/journal.pone.0154255.t002

the DR6 serological group (*DRB1*13* and *DRB1*14*, $P = 7.08X10^{-6}$, OR 0.49, 95%CI 0.36–0.67, Table 2). We further explored associations between *DRB1* alleles and SSc using RPE testing by sequential elimination of carriers of each allele with the strongest association (Table 2, right column). The prime strongest association was between SSc and *DRB1*14:06*, followed by **13:02*, **10:01*, **04:03*, and **04:07*. A protective association between the carrier frequency of *DQB1*03:01* ($P = 0.0009$, $Pc = 0.0150$, OR 0.56, 95% CI 0.40–0.79, Table 3) or *DPB1*02:01* ($P = 5.16X10^{-6}$, $Pc = 8.77X10^{-5}$, OR 0.52, 95% CI 0.39–0.69, Table 3) and SSc was detected. The associations between *DQB1* or *DPB1* alleles and SSc using RPE were also analyzed. RPE were tested by sequential elimination of carriers of each of the *DQB1* alleles *DQB1*03:01*, **06:04*, and **06:02*; *DPB1* alleles *DPB1*02:01* and **03:01*, respectively. No statistically significant predisposing associations were found for *DQB1*26 epi* ($P = 0.2443$, OR 1.24) or any HLA class II

Table 3. *HLA-DQB1* and *DPB1* allele carrier frequencies in the SSc patients and the healthy controls.

	Case (n = 463)	Control (n = 413)	*P*	OR	*Pc*	95%CI	*P* (RPE)
*DQB1*02:01*	1 (0.2)	2 (0.5)	0.6044	0.44	NS		
*DQB1*02:02*	7 (1.5)	3 (0.7)	0.3490	2.10	NS		
*DQB1*03:01*	67 (14.5)	96 (23.2)	0.0009	0.56	0.0150	(0.40–0.79)	0.0009
*DQB1*03:02*	112 (24.2)	83 (20.1)	0.1666	1.27	NS		
*DQB1*03:03*	124 (26.8)	111 (26.9)	1.0000	1.00	NS		
*DQB1*03:06*	1 (0.2)	0 (0.0)	1.0000	2.68	NS		
*DQB1*04:01*	91 (19.7)	86 (20.8)	0.6743	0.93	NS		
*DQB1*04:02*	51 (11.0)	29 (7.0)	0.0457	1.64	0.7312	(1.02–2.64)	
*DQB1*05:01*	79 (17.1)	44 (10.7)	0.0064	1.73	0.1030	(1.16–2.56)	
*DQB1*05:02*	16 (3.5)	16 (3.9)	0.8572	0.89	NS		
*DQB1*05:03*	31 (6.7)	34 (8.2)	0.4390	0.80	NS		
*DQB1*06:01*	185 (40.0)	144 (34.9)	0.1247	1.24	NS		
*DQB1*06:02*	54 (11.7)	65 (15.7)	0.0929	0.71	NS		0.0485
*DQB1*06:03*	4 (0.9)	6 (1.5)	0.5293	0.59	NS		
*DQB1*06:04*	30 (6.5)	50 (12.1)	0.0046	0.50	0.0741	(0.31–0.81)	0.0010
*DQB1*06:09*	3 (0.6)	6 (1.5)	0.3198	0.44	NS		
*DPB1*02:01*	128 (27.6)	175 (42.4)	$5.16X10^{-6}$	0.52	$8.77X10^{-5}$	(0.39–0.69)	$5.16X10^{-6}$
*DPB1*02:02*	39 (8.4)	33 (8.0)	0.9020	1.06	NS		
*DPB1*03:01*	65 (14.0)	35 (8.5)	0.0105	1.76	0.1793	(1.14–2.72)	0.0315
*DPB1*04:01*	31 (6.7)	41 (9.9)	0.0858	0.65	NS		
*DPB1*04:02*	92 (19.9)	65 (15.7)	0.1133	1.33	NS		
*DPB1*05:01*	272 (58.7)	250 (60.5)	0.6293	0.93	NS		
*DPB1*06:01*	9 (1.9)	3 (0.7)	0.1511	2.71	NS		
*DPB1*09:01*	124 (26.8)	82 (19.9)	0.0167	1.48	0.2844	(1.07–2.03)	
*DPB1*13:01*	28 (6.0)	20 (4.8)	0.4605	1.26	NS		
*DPB1*14:01*	11 (2.4)	8 (1.9)	0.8171	1.23	NS		
*DPB1*17:01*	4 (0.9)	3 (0.7)	1.0000	1.19	NS		
*DPB1*19:01*	5 (1.1)	2 (0.5)	0.4566	2.24	NS		
*DPB1*25:01*	0 (0.0)	1 (0.2)	0.4715	0.30	NS		
*DPB1*38:01*	2 (0.4)	0 (0.0)	0.5011	4.48	NS		
*DPB1*41:01*	3 (0.6)	2 (0.5)	1.0000	1.34	NS		
*DPB1*47:01*	1 (0.2)	0 (0.0)	1.0000	2.68	NS		
*DPB1*113:01*	0 (0.0)	1 (0.2)	0.4715	0.30	NS		

SSc: systemic sclerosis, OR: odds ratio, CI: confidence interval, *Pc*: corrected *P* value, NS: not significant, RPE: relative predispositional effects. Allele carrier frequencies are shown in parenthesis (%). Association was tested by Fisher's exact test using 2X2 contingency tables under the dominant model. RPE were tested by sequential elimination of carriers of each of the *DQB1* alleles *DQB1*03:01*, **06:04*, and **06:02; DPB1* alleles *DPB1*02:01* and **03:01*, respectively.

doi:10.1371/journal.pone.0154255.t003

alleles. Thus, lower carrier frequencies of the four class II alleles, *DRB1* 13:02*, *DRB1* 14:06*, *DQB1* 03:01*, and *DPB1* 02:01*, were present in SSc patients.

DRB1, *DQB1*, and *DPB1* alleles are in linkage disequilibrium. In order to elucidate which of the four protective alleles was responsible for the observed protective associations, conditional logistic regression analysis between them in SSc was performed (Table 4). The association of *DRB1* 13:02* remained significant, when conditioned on *DRB1* 14:06*, *DQB1* 03:01*, or *DPB1* 02:01*. Similarly, the association of *DRB1* 14:06* still remained significant, when conditioned on *DRB1* 13:02*, *DQB1* 03:01*, or *DPB1* 02:01*. The significant association of

Table 4. Conditional logistic regression analysis between the four protective HLA alleles in SSc.

HLA allele	Unconditioned		Conditioned on *DRB1*13:02*		Conditioned on *DRB1*14:06*		Conditioned on *DQB1*03:01*		Conditioned on *DPB1*02:01*	
	P	OR (95%CI)	$P_{adjusted}$	$OR_{adjusted}$ (95%CI)	$P_{adjusted}$	$OR_{adjusted}$ (95%CI)	$P_{adjusted}$	$OR_{adjusted}$ (95%CI)	$P_{adjusted}$	$OR_{adjusted}$ (95%CI)
*DRB1*13:02*	0.0006	0.48(0.31–0.73)	NA	NA	0.0005	0.47 (0.30–0.71)	0.0002	0.45 (0.29–0.69)	0.0006	0.47 (0.31–0.73)
*DRB1*14:06*	0.0046	0.05(0.01–0.41)	0.0040	0.05 (0.01–0.39)	NA	NA	0.0127	0.07 (0.01–0.57)	0.0046	0.05 (0.01–0.40)
*DQB1*03:01*	0.0013	0.59(0.43–0.82)	0.0005	0.56 (0.41–0.78)	0.0264	0.69 (0.50–0.96)	NA	NA	0.0019	0.60 (0.43–0.83)
*DPB1*02:01*	$8.91X10^{-6}$	0.58(0.46–0.74)	$8.71X10^{-6}$	0.58 (0.45–0.74)	$9.87X10^{-6}$	0.58 (0.45–0.74)	$1.22X10^{-5}$	0.58 (0.46–0.74)	NA	NA

SSc: systemic sclerosis, OR: odds ratio, CI: confidence interval, NA not applicable. *P*, OR, 95%CI, $P_{adjusted}$, and $OR_{adjusted}$ were calculated by logistic regression analysis under the additive model.

doi:10.1371/journal.pone.0154255.t004

*DQB1*03:01* was observed, when conditioned on *DRB1*13:02*, *DRB1*14:06*, or *DPB1*02:01*. The significant association of *DPB1*02:01* was also detected, when conditioned on *DRB1*13:02*, *DRB1*14:06*, or *DQB1*03:01*. Thus, significant protective associations for the four alleles with SSc were observed, when conditioned on each other, indicating an independent role for each protective allele in SSc.

When haplotype frequencies were compared between SSc patients and controls, a tendency for four haplotypes *(DRB1*15:01-DQB1*06:02-DPB1*02:01*, *DRB1*13:02-DQB1*06:04-DPB1*04:01*, *DRB1*04:06-DQB1*03:02-DPB1*02:01*, *DRB1*13:02-DQB1*06:04-DPB1*02:01*) to be protectively associated was observed (S1 Table). These four protective haplotypes include the abovementioned independent protective alleles.

HLA associations in SSc patients with ACA or ATA

We tested whether *HLA* class II alleles were associated with SSc with ACA. A significant association with susceptibility to SSc with ACA was found for the *DRB1*01:01* and *DRB1*10:01* alleles ($P = 0.0001$, $Pc = 0.0042$, OR 2.52, 95%CI 1.58–4.01; $P = 0.0003$, $Pc = 0.0097$, OR 11.17, 95%CI 2.42–51.48, respectively, S2 Table). A strong predisposing association between the carrier frequency of *DQB1*05:01* and SSc with ACA ($P = 1.18X10^{-6}$, $Pc = 1.89X10^{-5}$, OR 3.07, 95% CI 1.97–4.80, S3 Table) was detected. On the other hand, *DQB1*03:01* was associated with resistance to SSc with ACA ($P = 1.05X10^{-5}$, $Pc = 0.0002$, OR 0.32, 95%CI 0.18–0.55), despite the fact that this allele is known to be associated with susceptibility to SSc with ACA in Europeans [6]. *DPB1*04:02* was associated with SSc with ACA ($P = 0.0001$, $Pc = 0.0020$, OR 2.23, 95% CI 1.48–3.35, S4 Table). A tendency for the four class II alleles, *DRB1*13:02*, *DRB1*14:06*, *DQB1*03:01*, and *DPB1*02:01*, to be protectively associated with SSc with ACA was also observed (S2, S3 and S4 Tables). The similar tendency for the ACA associated alleles to be associated with lcSSc was observed (S2, S3 and S4 Tables), though it was weaker.

We then compared the allele carrier frequencies of *DRB1*, *DQB1*, and *DPB1* in SSc with ATA with their frequencies in healthy controls. A significant association with susceptibility to SSc with ATA was found for the *DRB1*15:02* allele ($P = 7.22X10^{-9}$, $Pc = 2.02X10^{-7}$, OR 3.58, 95%CI 2.33–5.50, S2 Table). A predisposing association between the carrier frequency of *DQB1*06:01* and SSc with ATA ($P = 3.20X10^{-5}$, $Pc = 0.0005$, OR 2.41, 95%CI 1.59–3.64, S3 Table) was detected. *DQB1*06:04* was associated with resistance to SSc with ATA ($P = 2.95X10^{-5}$, $Pc = 0.0004$, OR 0.06, 95%CI 0.01–0.45) and it was known that *DQB1*06:04* and protective *DRB1*13:02* are in strong linkage disequilibrium [26]. *DPB1*03:01* and *DPB1*09:01* are associated with SSc with ATA ($P = 3.42X10^{-5}$, $Pc = 0.0006$, OR 3.32, 95%CI

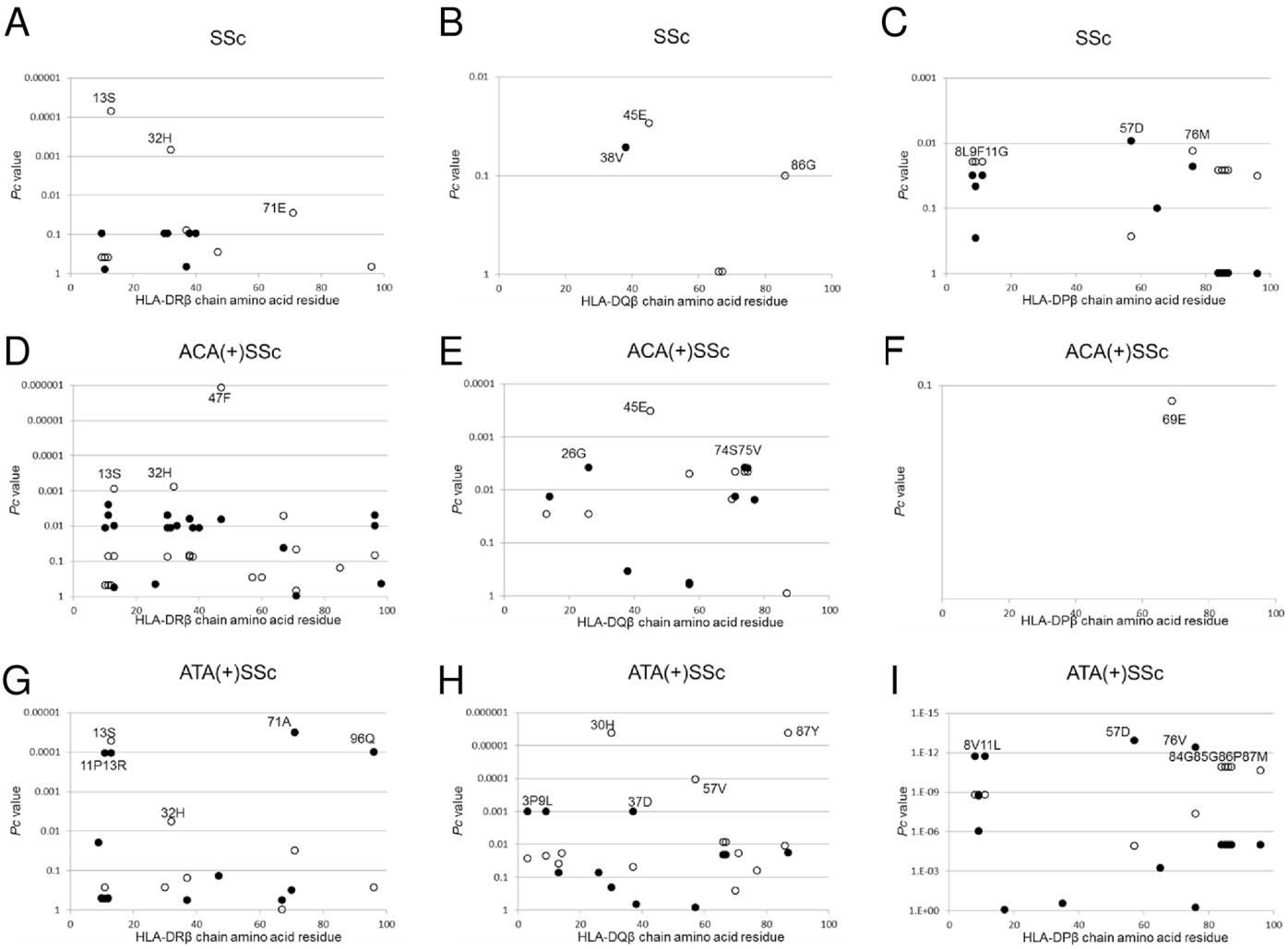

Fig 1. Associations of amino acid residues in the DRβ (A, D, G), DQβ (B, E, H), or DPβ (C, F, I) chain with SSc (A, B, C), anti-centromere antibody-positive [ACA(+)] SSc (D, E, F), and anti-topoisomerase I antibody-positive [ATA(+)] SSc (G, H, I). Corrected *P* (*Pc*) values were calculated by multiplying the *P* value by the number of amino acid residues tested. Associations were established by Fisher's exact test using 2X2 contingency tables. Predisposing associations are indicated by filled circles and protective associations by open circles.

doi:10.1371/journal.pone.0154255.g001

1.92–5.74; $P = 7.82X10^{-12}$, $Pc = 1.33X10^{-10}$, OR 4.54, 95%CI 2.94–7.01, respectively, S4 Table). A protective association for *DPB1*02:01* and *DPB1*04:01* was detected with SSc with ATA ($P = 2.06X10^{-8}$, $Pc = 3.50X10^{-7}$, OR 0.24, 95%CI 0.14–0.42; $P = 4.18X10^{-5}$, $Pc = 0.0007$, OR 0.04, 95%CI 0.00–0.62, respectively, S4 Table). A tendency for the four class II alleles, *DRB1*13:02*, *DRB1*14:06*, *DQB1*03:01*, and *DPB1*02:01*, to be protectively associated with SSc with ATA was observed (S2, S3 and S4 Tables). The similar tendency for the ATA associated alleles to be associated with dcSSc was observed (S2, S3 and S4 Tables). Thus, different predisposing associations of *DRB1*, *DQB1* or *DPB1* alleles were detected in SSc with ACA or ATA.

We tested whether *HLA* class II alleles were associated with ACA positive lcSSc, ACA negative lcSSc, ATA positive dcSSc, or ATA negative dcSSc (S5 Table). The predisposing or protective associations of *DRB1*, *DQB1* or *DPB1* alleles were mainly detected in ACA positive lcSSc or ATA positive dcSSc, suggesting that *HLA* class II alleles specifically influence the production of antibodies rather than the development of clinical subtypes of SSc.

Certain amino acid residues in the HLA-DRβ, DQβ, and DPβ chains are associated with SSc, or SSc with ACA or ATA

Finally, we analyzed the association with SSc with respect to each amino acid residue in the HLA-DRβ, DQβ, and DPβ chains. Serine at position 13 (13S, $P = 2.06X10^{-6}$, OR = 0.49, $Pc = 7.00X10^{-5}$, 95% CI 0.36–0.66) in the DRβ chain showed a strong protective association with SSc (Fig 1A, open circles). Glutamic acid at position 45 (45E, $P = 0.0009$, OR = 0.56, $Pc = 0.0291$, 95% CI 0.40–0.79) in the DQβ chain showed a protective association with SSc (Fig 1B, open circle), whereas aspartic acid at position 57 (57D, $P = 0.0005$, OR = 1.64, $Pc = 0.0092$, 95% CI 1.24–2.17) in the DPβ chain showed a predisposing association with SSc (Fig 1C, filled circle).

We also analyzed the association with SSc with ACA with respect to each amino acid residue in the HLA-DRβ, DQβ, and DPβ chains. Phenylalanine at position 47 (47F, $P = 3.43X10^{-8}$, OR = 0.37, $Pc = 1.17X10^{-6}$, 95% CI 0.26–0.53) in the DRβ chain showed strong protective associations with SSc with ACA (Fig 1D, open circles). Glutamic acid at position 45 (45E, $P = 1.05X10^{-5}$, OR = 0.32, $Pc = 0.0003$, 95% CI 0.18–0.55) in the DQβ chain showed protective associations with SSc with ACA (Fig 1E, open circle). No statistically significant association was found for amino acid residue in the DPβ chain.

In addition, we analyzed the association with SSc with ATA with respect to each amino acid residue in the HLA-DRβ, DQβ, and DPβ chains. Serine at position 13 (13S, $P = 1.52X10^{-6}$, OR = 0.29, $Pc = 5.16X10^{-5}$, 95% CI 0.17–0.50) in the DRβ chain showed a strong protective association with SSc with ATA (Fig 1G, open circle), whereas alanine at position 71 (71A, $P = 9.37X10^{-7}$, OR = 2.83, $Pc = 3.19X10^{-5}$, 95% CI 1.86–4.31) in the DRβ chain showed a strong predisposing association with SSc with ATA (Fig 1G, closed circle). Histidine at position 30 (30H, $P = 1.33X10^{-7}$, OR = 0.22, $Pc = 4.11X10^{-6}$, 95% CI 0.12–0.42), and tyrosine at position 87 (87Y, $P = 1.32X10^{-7}$, OR = 0.22, $Pc = 4.11X10^{-6}$, 95% CI 0.12–0.42) in the DQβ chain showed a protective association with SSc with ACA (Fig 1H, open circle). Aspartic acid at position 57 (57D, $P = 6.66X10^{-15}$, OR = 5.44, $Pc = 1.13X10^{-13}$, 95% CI 3.49–8.48), valine at position 76 (76V, $P = 2.20X10^{-14}$, OR = 5.27, $Pc = 3.74X10^{-13}$, 95% CI 3.39–8.18) in the DPβ chain showed a predisposing association with SSc (Fig 1I, filled circle). Thus, association analysis suggested roles for specific amino acid residues in the HLA-DRβ, DQβ, and DPβ chains.

Discussion

Several studies have noted predisposing associations of *HLA* class II alleles with SSc [6,8,9,13,14]. However, few studies for the *HLA* protective association have been validated in SSc. *DRB1*07:01*, *DRB1*15:01*, *DQB1*02:02*, and *DQB1*06:02* were protectively associated with European SSc [6], and *DRB1*01:01*, *DRB1*04:06*, *DRB1*07:01*, and *DPB1*02:01* were with Chinese SSc [13,14]. The present study reports significant protective associations of the four alleles, *DRB1*13:02*, *DRB1*14:06*, *DQB1*03:01*, and *DPB1*02:01*, with Japanese SSc (Table 2), though these protective associations except for *DPB1*02:01* were not observed in previous studies. A lower frequency of DR6 alleles in Asian patients with SSc has been reported, so far [9,13]. The protective effect of DR6 seems to be partly mediated by *DRB1*13:02*, which is a common protective allele for several autoimmune diseases in Japanese [21,22,27]. It was known that *DRB1*14:06 and DQB1*03:01* are in strong linkage disequilibrium in the Japanese population [26]. However, conditional logistic regression analysis between them in SSc revealed that they independently affected on the disease protection (Table 4). Because of the limited sample size of this study, the observed protective association was modest. The protective association of the four *HLA* class II alleles with SSc should be confirmed in future large scale studies.

In this study, we found a protective association of *DQB1*03:01* with Japanese SSc. This protective effect was also observed for the SSc with ACA (S3 Table). However, our findings are not

consistent with a previous report that *DQB1*03:01* is a risk allele for SSc with ACA in the European population [6]. This could be explained by the linkage disequilibrium of *DRB11-DQB1*03:01* in European populations [7]. However, we cannot rule out the possibility that there are other causative genes for SSc with ACA in the *HLA* region in linkage disequilibrium with the culprit gene in the *DQB1*03:01* haplotype. This possibility could be addressed by comparison of the re-sequencing data of the entire *HLA* region of *DQB1*03:01* haplotype in Japanese and Europeans.

The present study reports a significant predisposing association of *DRB1*01:01* and *DRB1*10:01*, *DQB1*05:01*, and *DPB1*04:02* with Japanese ACA positive SSc. The predisposing associations of *DRB1*10:01* and *DRB1*15:02* with ACA positive SSc in Chinese were reported [13]. On the other hand, *DRB1*01:01* and *DQB1*05:01* were associated with European ACA positive SSc [6]. The association of *DPB1*04:02* with Japanese ACA positive SSc was also reported [9]. Our findings are consistent with these previous reports. The higher haplotype frequency of *DRB1*01:01-DQB1*05:01* and *DRB1*10:01-DQB1*05:01* in the Japanese population suggests an important role of *DQB1*05:01* allele in the pathogenesis of ACA positive SSc in Japanese [26]. No associations with *DQB1*26 epi* including *DQB1*03:01* and *DQB1*06:01* were detected in the present study, suggesting that no *DQB1*26 epi* alleles other than *DQB1*05:01* are risk factors for ACA positive SSc in Japanese.

In the present study, the carrier frequencies of the *DRB1*15:02*, *DQB1*06:01*, *DPB1*03:01*, and *DPB1*09:01* alleles were higher in SSc patients with ATA. Associations between *DRB1*15:02*, *DQB1*06:01*, and *DPB1*09:01* and the presence of ATA have been reported in Japanese SSc patients [9]. The predisposing association of *DRB1*15:02*, *DRB1*16:02*, *DPB1*03:01* and *DPB1*13:01* with ATA positive SSc in Chinese was also reported [13,14]. *DRB1*11:04*, *DQB1*03:01*, and *DPB1*13:01* were strongly associated with ATA positive SSc in European descents [6]. The predisposing alleles in our study are overlapping with those reported in the previous reports.

We revealed that amino acid residues 13, 32 and 71 of the HLA-DRβ chain were protectively associated with SSc (Fig 1A). Amino acid residues 13, 32 and 71 form the HLA-DR peptide-binding groove [28], suggesting the involvement of peptide antigens bound to specific HLA-DR molecules in controlling the prevention of SSc. It was also found that other amino acid residues of the DRβ, DQβ, or DPβ chains were associated with SSc with ACA or ATA (Fig 1D–1I), though they are different from the results from the previous study in European populations [5]. These would be reflected by the ethnic differences of susceptible and protective *HLA* alleles [2,6,7]. This information suggests that peptide antigens loaded on specific HLA alleles controlled the generation of autoantibodies.

Because the distribution of *HLA* alleles in other ethnic populations is different from the Japanese, the role of some *HLA* class II alleles in SSc in other populations should be determined. Thus, the present study identified protective associations of *HLA* class II alleles with Japanese SSc; our findings support independent protective roles for the four class II alleles, *DRB1*13:02*, *DRB1*14:06*, *DQB1*03:01*, and *DPB1*02:01*, in the pathogenesis of SSc.

Supporting Information

S1 Fig. The statistical power in each condition of allele carrier frequency and odds ratio was calculated on the comparison between the overall SSc and the control.
(PDF)

S1 Table. HLA haplotype frequency in the SSc patients and controls. SSc: systemic sclerosis. Haplotypes with more than 1% frequency in controls are shown.
(PDF)

S2 Table. HLA-DRB1 allele carrier frequencies in the SSc subsets and the control. SSc: systemic sclerosis, dcSSc: diffuse cutaneous SSc, lcSSc: limited cutaneous SSc, ACA: anti-centromere antibodies, ATA: anti-topoisomerase antibodies, OR: odds ratio, CI: confidence interval, Pc: corrected P value, NS: not significant. Allele carrier frequencies are shown in parenthesis (%). Association was tested between the SSc subsets and the control by Fisher's exact test using 2X2 contingency tables under the dominant model.
(PDF)

S3 Table. HLA-DQB1 allele carrier frequencies in the SSc subsets and the control. SSc: systemic sclerosis, dcSSc: diffuse cutaneous SSc, lcSSc: limited cutaneous SSc, ACA: anti-centromere antibodies, ATA: anti-topoisomerase antibodies, OR: odds ratio, CI: confidence interval, Pc: corrected P value, NS: not significant. Allele carrier frequencies are shown in parenthesis (%). Association was tested between the SSc subsets and the control by Fisher's exact test using 2X2 contingency tables under the dominant model.
(PDF)

S4 Table. HLA-DPB1 allele carrier frequencies in the SSc subsets and the control. SSc: systemic sclerosis, dcSSc: diffuse cutaneous SSc, lcSSc: limited cutaneous SSc, ACA: anti-centromere antibodies, ATA: anti-topoisomerase antibodies, OR: odds ratio, CI: confidence interval, Pc: corrected P value, NS: not significant. Allele carrier frequencies are shown in parenthesis (%). Association was tested between the SSc subsets and the control by Fisher's exact test using 2X2 contingency tables under the dominant model.
(PDF)

S5 Table. HLA class II allele carrier frequencies in the SSc subsets and the control. SSc: systemic sclerosis, dcSSc: diffuse cutaneous SSc, lcSSc: limited cutaneous SSc, ACA: anti-centromere antibodies, ATA: anti-topoisomerase antibodies, OR: odds ratio, CI: confidence interval, Pc: corrected P value, NS: not significant. Allelecarrier frequencies are shown in parenthesis (%). Association was tested between the SSc subsets and the control byFisher's exact test using 2X2 contingency tables under the dominant model.
(PDF)

Acknowledgments

We thank Ms. Mayumi Yokoyama (Clinical Research Center for Allergy and Rheumatology, Sagamihara Hospital) and Ms. Tomomi Hanawa (Clinical Research Center for Allergy and Rheumatology, Sagamihara Hospital) for secretarial assistance.

Author Contributions

Conceived and designed the experiments: HF ST NT. Performed the experiments: HF SO A. Kawasaki. Analyzed the data: HF. Contributed reagents/materials/analysis tools: HF K. Shimada S. Sugii TM AH A. Komiya NF KK A. Osada AI YK TN K. Setoguchi Akiko Okamoto Akira Okamoto NC ES HK MK SH TS KM MH MF S. Sato KT SN ST. Wrote the paper: HF ST NT.

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