

HLA class II genes associated with anticentromere antibody in Japanese patients with systemic sclerosis (scleroderma)

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Abstract

Objective—To define further HLA class II gene associations with anticentromere antibody (ACA), a major serum antinuclear antibody in patients with systemic sclerosis (SSc).

Methods—HLA class II genes were determined using polymerase chain reaction/restriction fragment length polymorphisms in 94 Japanese patients with SSc (22 ACA positive and 72 ACA negative) and 50 race matched normal control subjects.

Results—Frequency of DQB1*0501 was increased in ACA positive SSc patients compared with ACA negative SSc patients (36% versus 13%; $p = 0.02$, odds ratio = 4.0, 95% confidence interval 1.1 to 13.9), but the association of ACA with a polar amino acid at position 26 in the DQB1 β 1 domain, which was demonstrated in white North Americans, was not observed in Japanese. The DRB1*0101, *0405, and *1302 alleles were associated with high ACA titres, whereas DRB1*1502 was associated with low ACA titres and a low frequency of centromere protein C (CENP-C) reactivity.

Conclusions—These results suggest that the ACA response is associated with multiple HLA class II genes and that ACA positive SSc patients are heterogeneous in terms of immunogenetic background.

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It is well known that many autoimmune diseases are associated with certain HLA types. Systemic sclerosis (or scleroderma) (SSc) is an autoimmune disease characterised by fibrosis of the dermis and internal organs and microvascular damage.¹ However, previous studies have failed to detect any reproducible HLA associations with SSc,² presumably because of the heterogeneity of the disease. Recent molecular analyses of genes encoding HLA class II molecules have revealed that HLA class II genes are associated more closely with each SSc related serum antinuclear antibody (ANA) than with the disease itself. For example, the presence of antibody to DNA topoisomerase I (topo I) is associated with DRB1*1502 and DQB1*0601 in Japanese, and broad specificities of DQ3 (DQB1*0301–*0303) in black and white North Americans.^{3 4}

Anticentromere antibody (ACA) is one of the major SSc related serum ANAs and is detected

in about 20% of SSc patients from various ethnic backgrounds.⁵ ACA occurs in a relatively homogeneous SSc subset having limited cutaneous SSc or the CREST variant (calcinosis, Raynaud's phenomenon, oesophageal dysmotility, sclerodactyly, telangiectasia).¹ ACA in SSc sera mainly recognise three major centromere constituent proteins (CENP) of molecular weights 16 kDa (CENP-A), 80 kDa (CENP-B), and 140 kDa (CENP-C).⁶ Increased frequencies of HLA-DR1, -DR4, and -DR8 have been detected in SSc patients with ACA by serological HLA typing.^{7 8} The association of ACA with DR1 and DR4 was confirmed by a recent study using molecular HLA typing.⁹ Another study using restriction fragment length polymorphism analysis of genomic DNA reported increased frequencies of DR11 and DQ7 (DQB1*0301) in patients with the CREST variant, the majority of whom were considered to have ACA.¹⁰ A large study using HLA class II genotyping showed that all the 42 ACA positive SSc patients, compared with 69% of ACA negative SSc patients and 71% of race matched normal control subjects, had at least one DQB1 allele with a polar amino acid (glycine or tyrosine) at position 26 in the β 1 domain, which is shared by DQB1*0501–*0504, *0601, *0301, *0401, and *0402.¹¹ This DQB1 gene association was confirmed by one study analysing HLA class II alleles in ACA positive SSc patients and their relatives,¹² but not by another study.¹³

In this study, we analysed the HLA class II gene associations with ACA in Japanese patients with SSc. We also examined HLA associations with ACA titres and immunoreactivities against individual CENP (CENP-A, -B, and -C).

Patients and methods

PATIENTS AND CONTROLS

We studied 94 unrelated Japanese SSc patients who were seen for evaluation between October 1992 and April 1993 at either Keio University School of Medicine (Tokyo) (90 patients) or Nippon Kokan Hospital (Kawasaki, adjacent to Tokyo) (four patients). All satisfied the American College of Rheumatology (formerly the American Rheumatism Association) preliminary classification criteria for SSc.¹⁴ Nineteen, 54, and 21 patients were classified as having diffuse cutaneous SSc, limited cutaneous SSc, and SSc overlap variant, respectively, according to the classification described by Medsger.¹ Twenty one patients with SSc

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overlap included six with SSc-systemic lupus erythematosus (SLE), seven with SSc-polymyositis (PM), three with SSc-rheumatoid arthritis (RA), four with SSc-SLE-PM, and one with SSc-PM-RA. Fifty healthy Japanese volunteers living in the Tokyo-Kawasaki area were selected as race matched normal control subjects.

ANA ASSAYS

Serum samples for analysis were obtained from all SSc patients at the time of their first visit and stored at -20°C . To identify SSc related ANAs, we performed indirect immunofluorescence on commercially available HEP-2 cell slides and HeLa cell chromosomal spread slides (MBL, Nagano, Japan), double immunodiffusion using calf and rabbit thymus extract as an antigen, and immunoprecipitation assays using ^{32}P - and ^{35}S -labelled HeLa cell extracts as described elsewhere.¹⁵ ACA was defined as positive if immunofluorescence using serum samples at a dilution of 1:40 showed a discrete speckled pattern consistent with ACA on HEP-2 cells, and two small spheres located on the centromeric region of each chromosome on HeLa cell chromosomal spreads.¹⁶ Serum ACA titres were determined by indirect immunofluorescence on chromosomal spreads using twofold serial dilutions of sera ranging from 1:200 to 1:6400. Immunoreactivities against CENP-A, -B, and -C were examined in ACA positive sera by immunoblots using mitotic chromosomes from colcemid arrested HeLa cells as antigens.¹⁷ Briefly, HeLa mitotic chromosomes were resolved on 11% polyacrylamide-sodium dodecyl sulphate gels and transferred onto nitrocellulose filters. The filters were then probed with sera diluted at 1:500 and subsequently with horseradish peroxidase conjugated goat antihuman IgG (Fc γ specific, GIBCO BRL, Grand Island, NY). Immunoreactivities were visualised using a chemiluminescence detection system (DuPont NEN, Boston, MA).

HLA CLASS II ALLELE TYPING

The HLA-DRB1, -DQB1, and -DPB1 genes were typed using restriction fragment length polymorphisms of polymerase chain reaction amplified genomic DNA as described previously.¹⁸ The numbers of alleles defined by this method are 44 (DRB1), 13 (DQB1), and 19 (DPB1).

STATISTICAL ANALYSIS

Differences in frequencies were analysed by Fisher's two tailed exact test. Corrected *p* values were obtained by multiplying observed *p* values by the number of comparisons made (DRB1 \times 44, DQB1 \times 13, and DPB1 \times 19). When an association with a particular specificity had been reported previously, the *p* value was not corrected.¹¹ The odds ratio (OR) with a 95% confidence interval (95% CI) of the significant differences was calculated, and

when one of the four cells of the 2×2 tables was 0, Haldane's modification was applied.

Results

Twenty two (23%) of 94 SSc patients were identified as having ACA. Coexistence of other SSc related ANAs in ACA positive SSc patients was infrequent; only one had antibody to Th ribonucleoprotein (RNP). All 22 ACA positive patients were classified as having limited cutaneous SSc. Among 72 ACA negative SSc patients, anti-U1 RNP was detected in 34, anti-topo I in 30, anti-RNA polymerase in four, anti-Th RNP in four, anti-U3 RNP (fibrillar) in three, and anti-Ku in three. The distribution of SSc related ANAs was similar to that reported in our previous study involving 275 unselected Japanese patients with SSc.⁵

We could not find any statistically significant differences in HLA-DR allele frequencies when HLA class II gene frequencies in 22 ACA positive patients were compared with those in 72 ACA negative patients and in 50 normal control subjects. However, DR1 (DRB1*0101) was more frequent in ACA positive patients than in ACA negative patients and normal controls (32% versus 13% and 12%, *p* = 0.051 and 0.09, respectively). In contrast, there were trends toward lesser frequencies of DR5 (all combined DRB1*1101, *1102, *1201, and *1202) and DR9 (DRB1*0901) in ACA positive patients compared with the other two groups (5% versus 19% and 16%; and 9% versus 24% and 30%, respectively). When DQB1 allele frequencies were compared between ACA positive SSc patients and ACA negative SSc patients or healthy controls (table 1), DQB1*0501 was more frequent in ACA positive patients than in ACA negative patients (*p* = 0.02). There were

Table 1 HLA-DQB1 and DPB1 allele frequencies in 94 Japanese patients with systemic sclerosis (SSc) with or without anticentromere antibody (ACA), and 50 race matched normal controls

Alleles	SSc patients with ACA (n = 22)	SSc patients without ACA (n = 72)	Normal controls (n = 50)
DQB1*0501	8 (36)*	9 (13)	8 (16)
DQB1*0502	2 (9)	3 (4)	3 (6)
DQB1*0503	1 (5)	2 (3)	8 (16)
DQB1*0601	9 (41)	39 (54)	18 (36)
DQB1*0602	2 (9)	3 (4)	5 (10)
DQB1*0604	4 (18)	4 (6)	7 (14)
DQB1*0301	1 (5)†	19 (26)	13 (26)
DQB1*0302	6 (27)	22 (31)	7 (14)
DQB1*0303	2 (9)‡	16 (22)	16 (32)
DQB1*0401	5 (23)	9 (13)	10 (20)
DQB1*0402	1 (5)	5 (7)	4 (8)
DPB1*0201	4 (18)	18 (25)	18 (36)
DPB1*0202	4 (18)	2 (3)	5 (10)
DPB1*0301	1 (5)	11 (15)	5 (10)
DPB1*0401	1 (5)	2 (3)	4 (8)
DPB1*0402	12 (55)§	15 (21)	11 (22)
DPB1*0501	11 (50)	39 (54)	30 (60)
DPB1*0901	5 (23)	33 (46)	8 (16)
DPB1*1301	0	1 (1)	2 (4)
DPB1*1401	0	1 (1)	0

Values are number (%).

**p* = 0.02, OR = 4.0 (95% CI 1.1 to 13.9) compared with SSc patients without ACA; †*p* = 0.04, OR = 0.13 (95% CI 0.0 to 1.0) compared with SSc patients without ACA, and *p* = 0.050, OR = 0.14 (95% CI 0.0 to 1.1) compared with normal controls. ‡*p* = 0.04, OR = 0.21 (95% CI 0.02 to 1.1) compared with normal controls. §*p* = 0.005, OR = 4.6 (95% CI 1.5 to 14.2) compared with SSc patients without ACA, and *p* = 0.01, OR = 4.3 (95% CI 1.3 to 14.2) compared with normal controls.

trends toward negative associations of ACA with DQB1*0301 and DQB1*0303. The frequency of DPB1*0402 was greater in ACA positive patients compared with ACA negative patients and normal control subjects ($p = 0.005$ and 0.01 , respectively), but this difference did not reach statistical significance after correlation of the p value. The DQB1 alleles having a polar amino acid at position 26, which were reported to be essential for the ACA response in white North American patients,¹¹ were found in all except one ACA positive patients (95%), compared with 63 (88%) of 72 ACA negative patients (NS) and 44 (88%) of 50 normal controls (NS).

As all 22 ACA positive patients were classified as having limited cutaneous SSc, HLA class II gene frequencies in ACA positive SSc patients were compared with those in 32 ACA negative patients with limited cutaneous SSc. Frequencies of DQB1*0501 and DPB1*0402 were again greater (36% versus 13%, $p = 0.050$, OR = 4.0, 95% CI 0.9 to 20.9; and 50% versus 22%, $p = 0.01$, OR = 4.3, 95% CI 1.1 to 16.7, respectively) and that of DQB1*0301 was again less (5% versus 38%, $p = 0.008$, OR = 0.08, 95% CI 0.0 to 0.7) in ACA positive compared with ACA negative patients with limited cutaneous SSc. Exclusion of 21 patients with SSc overlap did not influence the HLA-ACA associations described above (data not shown).

Table 2 summarises the DRB1 alleles, and the ACA titres and reactivities against CENP-A, -B, and -C, in 22 ACA positive SSc patients. ACA titres ranged from 200 to ≥ 6400 . When they were separated into high ACA titres ($n = 14$, ≥ 1600) and low ACA titres ($n = 8$, < 1600), six of seven patients having DRB1*0101, four of five patients having DRB1*0405, and all four patients having DRB1*1302 were included in the high ACA titre group. One of these three alleles was detected in 13 (93%) of 14 patients with high ACA titres, compared with two (25%) of eight patients with low ACA titres ($p = 0.002$, OR = 39, 95% CI 2.1 to 1924). In contrast,

five of six patients possessing DRB1*1502 were included in the low ACA titre group, and the remaining one DRB1*1502 positive patient, whose ACA titre was 3200, had DRB1*1302. Therefore, the frequency of DRB1*1502 in patients with high ACA titres (7%) was significantly lower than that in patients with low ACA titres (68%; $p = 0.01$, OR = 0.05, 95% CI 0.0 to 0.7). No significant associations between ACA titres and DQB1 and DPB1 alleles were found.

CENP-A, -B, and -C were recognised by 22 (100%), 22 (100%), and 19 (86%) sera, respectively. All three patients negative for CENP-C reactivity had DRB1*1502, and CENP-C reactivity was detected in only three (50%) of six patients with DRB1*1502 compared with all 16 patients lacking DRB1*1502 ($p = 0.01$, OR = 0.03, 95% CI 0.0 to 0.7). DQB1*0601 in linkage disequilibrium with DRB1*1502 is probably not responsible for this negative association with CENP-C reactivity, as sera from all three ACA positive patients possessing DRB1*0803 (another major DRB1 allele that links to DQB1*0601 in Japanese) were reactive with CENP-C.

Discussion

We examined HLA-DRB1, -DQB1, and -DPB1 gene associations with ACA in Japanese patients with SSc. To minimise patient selection bias, we recruited all patients seen in clinic during a period of seven months. As a result, the number of ACA positive SSc patients was relatively small ($n = 22$); however, we believe that the possible consequent bias does not affect our conclusion, as ANA distribution in the present study was similar to that in unselected SSc patients in our previous study.⁵ DQA1 was not examined in this study because of strong linkage disequilibrium between DQA1 and DQB1 genes. Our results can be summarised as follows: the presence of ACA was associated with DQB1*0501; a low frequency of CENP-C reactivity was associated with DRB1*1502; high ACA titres were

Table 2 Anticentromere antibody (ACA) titres and reactivities against three centromere constituent proteins (CENP-A, -B, and -C) in 22 ACA positive SSc patients, and their DRB1 alleles

Pt	ACA No titre	CENP reactivity			HLA-DR											
		A	B	C	DR1	DR2			DR4	DR5	DR6		DR8		DR9	
					0101	1501	1502	1602	0405	0401,3,6	1101	1302	1403,5	0802	0803	0901
1	6400 ≤	+	+	+	●											●
2	6400 ≤	+	+	+	●			●	●							
3	3200	+	+	+	●	●			●							
4	3200	+	+	+	●				●							
5	3200	+	+	+	●				●							
6	3200	+	+	+	●				●							●
7	3200	+	+	+	●				●							
8	3200	+	+	+	●				●			●				
9	3200	+	+	+	●				●			●				
10	3200	+	+	+	●				●			●				
11	3200	+	+	+	●				●			●			●	
12	3200	+	+	+	●				●			●			●	
13	3200	+	+	+	●				●			●			●	
14	1600	+	+	+	●				●			●			●	

15	800	+	+	+	●				●							
16	800	+	+	-	●				●							
17	800	+	+	+	●				●							
18	400	+	+	+	●	●			●							
19	400	+	+	+	●				●				●			
20	400	+	+	-	●				●						●	
21	200	+	+	-	●				●							
22	200	+	+	+	●				●				●			

positively associated with DRB1*0101, DRB1*0405, and DRB1*1302, and negatively associated with DRB1*1502. These results suggest that the ACA response is associated with several alleles located on HLA-DR and -DQ genes, though some of the associations could be explained by the known linkage disequilibrium between DRB1 and DQB1. Furthermore, our data indicate that HLA class II genes regulate antibody responses by means of antibody titres and immunoreactivities against the constituent proteins. This immune regulatory mechanism is analogous to that of other SSc related ANAs (including anti-topo I and anti-U1 RNP responses) that we have shown previously.^{3 19}

We examined the HLA associations with reactivities to CENP, but only a borderline association with CENP-C reactivity was detected. One possible reason for the weak associations is high frequencies of CENP-A, -B, and -C reactivities in ACA positive SSc patients; all ACA positive sera recognised CENP-A and -B, and 19 of 22 sera were reactive with CENP-C. Further study including a large number of ACA positive patients is necessary to confirm possible associations between HLA class II alleles and reactivities to CENP. We have shown previously that reactivities against different B cell epitopes on the topo I molecule were associated with different HLA-DR specificities.³ As CENP were suggested to carry multiple epitopes recognised by patients' sera,²⁰ detailed analyses of B cell epitopes on the CENP are helpful in further defining HLA class II gene associations with ACA.

Previous studies analysing HLA associations with ACA proposed that the HLA-DQ genes primarily regulate ACA responses based on shared amino acid sequences that are present in the DQB1 alleles associated with ACA.^{11 13} Reveille *et al* reported that ACA was associated with DQB1*0501 and *0301 in white North Americans and that the DQB1 alleles possessing a polar amino acid at position 26 were detected in all ACA positive SSc patients examined.¹¹ The increased frequency of DQB1*0501 in ACA positive SSc patients is consistent with our results, whereas the frequency of DQB1*0301 was decreased in ACA positive SSc patients in this study. Furthermore, we did not detect an association between ACA and a polar amino acid at position 26 in the β 1 domain of DQB1. It is unlikely that these differences between Reveille's results and ours reflect ethnic or geographic differences between the respective subjects studied, as Morel *et al*, analysing the same North American population as was examined by Reveille, also reported that 10 (21%) of 47 ACA positive SSc patients lacked the polar amino acid at position 26 in the DQB1 genes.¹³ Morel's group described, instead, an increased frequency of the TRAELEDT sequence at amino acids 71–77 of the DQB1 chain in ACA positive SSc patients compared with ACA negative SSc patients lacking DQB1*0501 (78% versus 62%).¹³ However, the association of ACA with the TRAELEDT sequence in DQB1*0501 negative individuals was not confirmed in our study,

because the TRAELEDT sequence was detected in 13 (93%) of 14 ACA positive SSc patients, but also in all 63 ACA negative SSc patients and 39 (93%) of 42 normal controls, who did not possess DQB1*0501 (data not shown). In common with these recent studies, our present report suggests that ACA responses cannot be explained by the shared amino acid sequences present in the DQB1 genes.

We have shown instead, that high ACA titres are associated with multiple DRB1 alleles, including DRB1*0101, *0405, and *1302. These DRB1 alleles do not have shared amino acid sequences in the antigen binding domain. Our recent studies of T cells reactive with topo I revealed that distribution of T cell epitopes on topo I had an association with the nature of the patient's HLA-DR alleles,²² suggesting that each T cell epitope is restricted by distinct HLA-DR alleles. Although to date there are no reports describing T cell responses to centromere proteins, it is possible that several T cell epitopes are present on the centromere antigen complex and that each T cell epitope is restricted by different HLA class II molecules.

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