

# Association of CTLA-4 with systemic sclerosis in Japanese patients

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## Abstract

### Objective

*The contribution of CTLA-4 alleles to the pathogenesis of systemic sclerosis (SSc) was studied in Japanese patients.*

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### Methods

*CTLA-4 typing in 2 dimorphic sites, +49 A/G and -308 C/T, was carried out in 62 SSc patients and 107 normal subjects by the PCR-RFLP (restriction fragment length polymorphism) method. HLA-DRB1\*15 and \*08 genotyping were carried out by the PCR-SSCP (simple-stranded DNA conformation polymorphism) method.*

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### Results

*In SSc the frequency of the +49A allele increased slightly (40.3%), but was not significant. In SSc with diffuse scleroderma and SSc with anti-topoisomerase I antibody, the +49A also increased (43.8%, and 48.0%, respectively) but again was not significant. A significant increase in the +49A was not observed in SSc with HLA-DRB1\*1502 or ORB1\*0802. In contrast, the +49A had significantly increased in SSc with the anti-RNP antibody [52.9%,  $p = 0.0337$ , Odds ratio (OR) = 2.27 (95% confidential interval (CI) = 1.09 - 4.71)]. HLA-DRB1\*1502 and \*0802 had no influence on the association of anti-RNP antibody with the +49A. The +49AA genotype increased significantly in SSc without lung fibrosis [31.8%,  $p = 0.0456$ , OR = 3.37 (CI = 1.16 - 9.87)], especially in limited SSc without lung fibrosis [33.3%,  $p = 0.0319$ , OR = 3.62 (CI = 1.16 - 11.29)]. The dimorphism at -308 did not associate with SSc.*

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### Conclusion

*In Japanese scleroderma, the +49A allele of CTLA-4 increased in the presence of SSc with the anti-RNP antibody.*

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### Key words

CTLA-4, RNP, SSc, lung fibrosis, Japanese.

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## Introduction

Systemic sclerosis (SSc) is a well known autoimmune disease characterized by various clinical features such as proximal scleroderma, bilateral pulmonary fibrosis, and auto-antibodies. For classification, two major types, SSc with diffuse scleroderma and SSc with limited scleroderma (1, 2), were clinically defined. Although both the former and the latter showed associations with anti-topoisomerase I antibody (a-Scl-70) and anti-centromere antibody (ACA), respectively, these auto-antibodies did not exactly match the clinical types of the disease.

The possible contribution of genetic factors to SSc has been presented in several studies, though the etiology of SSc is still unclear. In Japanese, HLA DRB1\*1502 - DRB5\*0102 haplotype and DRB1\*0802 were found to be associated with diffuse scleroderma and a-Scl-70-positive SSc (3, 4). Reveille *et al.* showed an association between ACA and polar amino acid residue at position 26 of HLA-DQB1 molecules (5), and associations of HLA-DR5 (DRB1\*1101, 1104) and uncharged polar amino acid residue at position 30 with a-Scl-70-positivity were also found (6).

Cytotoxic T lymphocyte associated-4 (CTLA-4) and CD28 on T cells, on the other hand, bind to CD80 and CD86 (7), and CTLA-4 is a negative regulator of T cell activation (8). The ligation of CTLA-4 blocks CD-28 dependent T cell activation and IL-2 synthesis (9). The CTLA-4 molecule was thought to terminate the immune response by CD28 and to keep homeostatic balance of the immune system. CTLA-4 has also been reported to regulate negatively T-cell function (10). Therefore, CTLA-4 would appear to be an important negative regulator of autoimmune diseases (11). In experimental allergic encephalomyelitis, inhibition of CTLA-4 was reported to block enhancement of the clinical disease (12).

The CTLA-4 gene is located on chromosome 2q33 and dimorphisms are reported in exon 1 and in the promoter region (13,14). The former is a substitution of adenine for guanine (+49A/G) (13) and the latter is a substitution of

cytosine for thymine (-318C/T) (14). Associations of CTLA-4 (+49A/G) with systemic lupus erythematosus (SLE) (15, 16), insulin dependent diabetes mellitus (IDDM) (17, 18), Graves' disease (13, 18), Hashimoto's thyroiditis (19) and multiple sclerosis (20) have been reported, though the results are sometimes controversial. Heward *et al.* reported no association of the +49A/G dimorphism with SLE (21). In rheumatoid arthritis (RA), Seidl *et al.* reported an association of the +49GG genotype with RA patients carrying HLA-DRB1\*0401 (22), although Barton *et al.* could not find any association between the CTLA-4 +49A/G dimorphism and RA (23).

For the pathogenesis of SSc, the contribution of immunological abnormality was assumed. In the salivary glands of very early stage SSc, expression of TNF has been observed prior to the onset of skin change (24), and Koch *et al.* reported an increased expression of cytokines and cellular adhesion molecules in the skin of patients with systemic sclerosis (25).

Considering the immune-regulatory function of CTLA-4, CTLA-4 gene is an interesting candidate as a disease-susceptible gene or a genetic marker. We studied 62 Japanese SSc patients to clarify the contribution of CTLA-4 genes to the disease using the PCR-RFLP method (14, 18). The associations of CTLA-4 with auto-antibody, disease type, and susceptible DRB1\*1502 (3, 4) gene were examined and discussed.

## Patients and methods

### Patients

To investigate how SSc is associated with CTLA4, 62 unrelated Japanese patients aged  $52.5 \pm 11.3$  years old (mean  $\pm$  SD) (60 women and 2 men) were studied. All patients with SSc fulfilled the criteria outlined by the American Rheumatism Association (ARA) (1). As controls, 107 randomly selected, unrelated, healthy subjects were compared. Familial analysis was not available in this study for either patients or controls.

In the SSc group, 25 patients (40.3%) aged  $53.3 \pm 8.8$  were positive for a-Scl-

**Table I.** Clinical features in SSC.

	Whole SSC	Diffuse	Limited	a-Scl-70+	ACA+
No. of cases	62	24	38	25	12
Age	52.5±11.3 <sup>a</sup>	51.0±9.4	53.5±12.3	53.3±8.8	60.4±14.4
Age at onset	40.7±13.1	37.7±11.2	42.5±13.9	40.4±10.1	51.8±15.4
Duration	11.8±7.2	13.2±6.6	10.9±7.5	12.9±7.5	8.5±5.6
Male	2 (3.2) <sup>b</sup>	0	2 (5.3)	0	0
Scl-70	25 (40.3)	18 (75.0)	7 (18.4)		0
ACA	12 (19.4)	0	12 (31.6)	0	

a: Mean ± SD; b: %.

70 and 12 patients (19.4%) aged  $60.4 \pm 14.4$  were positive for ACA. Twenty four patients (38.7%) aged  $51.0 \pm 9.4$  had SSc with diffuse scleroderma and 38 patients (61.3%) aged  $53.5 \pm 12.3$  had SSc with limited scleroderma (2). Positive results for a-Scl-70 and ACA were 18 (75.0%) and 0 (0%) in diffuse scleroderma, respectively, and 7 (18.4%) and 12 (31.6%) in limited scleroderma, respectively (Table I).

The frequencies of anti-U1-RNP (a-RNP) were 27.4%, 16.7% and 34.2% in whole SSc, diffuse scleroderma and limited scleroderma, respectively. The frequencies of anti-SSA (a-SS-A) were 33.9%, 33.3% and 34.2% in each group respectively. Only 2 patients showed positive results for anti-SS-B (a-SS-B).

#### Detection of autoantibodies

Anti-Scl-70, a-RNP, a-SS-A and a-SS-B autoantibodies were detected by the ELISA (enzyme-linked immunosorbent assay) method using MESA-CUP-2 TEST Scl70, MESACUP-2 TEST RNA, MESACUP-2 TEST SSA, and MESACUP-2 TEST SSB (MBL, Nagoya, Japan), respectively. ANA was detected by an indirect immunofluorescent method using Hep-2 cell specimens as nuclear antigens (Quantafluor Test Kit (Hep-2), Kallestad, Chaska, USA). ACA was recognized as a characteristic discrete speckled nuclear staining pattern. These methods are standard in the Central Laboratory Service of Tokyo University Hospital (26).

#### CTLA-4 genotyping

The dimorphism at position +49 in exon 1 (+49A/G) was detected by the PCR-RFLP method of Donner *et al.* using specific oligonucleotide primers,

5'-GCTCTACTTCCTGAAGACCT-3' and 5'-AGTCTCACTCACCTTTG-CAG-3'. PCR was performed under the following conditions: initial denaturation for 4 min at 94°C, annealing for 45s at 58°C, extension for 45s at 72°C, denaturation for 45s at 94°C (30 cycles), and a final extension for 10 min at 72°C. The PCR product was digested using Bbv1 at 37°C for 16 hours (18). The dimorphism at position -318 in the promoter region (-308C/T) was examined by the PCR-RFLP method of Deichmann *et al.*, using oligonucleotide primers, 5'-AAATGAATTG-GACTGGATGGT-3' and 5'-TTACGA-GAAAGGAAGCCGTG-3'. PCR was performed under the following conditions: initial denaturation for 5 min at 94°C, annealing for 45s at 60°C, extension for 60sec at 72°C and denaturing for 45s at 94°C (35 cycles), and final extension for 10 min at 72°C. The PCR product was digested using MseI at 37°C for 2 hours (14).

#### HLA DR typing

HLA-DR typing was performed on the

second exon of HLA-DRB1 by the PCR method using the specific primers described previously (3).

#### Genotyping of HLA-DR15 and 16, and DR8

HLA-DR15, 16 and 8, all of which have been shown to be associated with Japanese SSc (3, 4), were genotyped by the PCR-SSCP (single-stranded DNA conformation polymorphism) method using the specific primers described previously (3). SSCP fragments suspended in gel were detected by silver staining (Daiichi Kagaku, Japan) (3).

#### Statistics

Fisher's exact test was used for comparison (3). Odds ratio (OR) and 95% confidential interval (CI) were shown occasionally (3).

#### Results

Genotype frequencies of the +49A/G dimorphism are shown in Table II. In the whole SSc group, the +49AA had slightly but not significantly increased (19.4% Vs 12.1% in control). In SSc with diffuse scleroderma, the frequency of the +49AA increased slightly, but the increase was not significant (20.8%). The +49AA also increased slightly in SSc with limited scleroderma (18.4%). In SSc with a-Scl-70, the prevalence of the +49AA had also slightly increased (20.0%), but again the difference was not significant. The prevalence of the +49AA had also slightly increased (33.3%) in patients with ACA, but the increase was not significant. Allele frequency of the A at +49 had increased in

**Table II.** CTLA-4 (+49 A/G) Bbv1 RFLP in SSC.

No. of Cases	Normal 107	Whole SSC 62	Diffuse 24	Limited 38	a-Scl-70+ 25	ACA+ 12
Genotype freq.						
AA	13 (12.1) <sup>a</sup>	12 (19.4)	5 (20.8)	7 (18.4)	5 (20.0)	4 (33.3)
AG	45 (42.1)	26 (41.9)	11 (45.8)	15 (39.5)	14 (56.0)	3 (25.0)
GG	49 (45.8)	24 (38.7)	8 (33.3)	16 (42.1)	6 (24.0)	5 (41.7)
Phenotype freq.						
A	58 (54.2)	38 (61.3)	16 (66.7)	22 (57.9)	19 (76.0)	7 (58.3)
G	94 (87.8)	50 (80.6)	19 (79.2)	31 (81.5)	20 (80.0)	8 (66.7)
Allele freq.						
A	71 (33.2)	50 (40.3)	21 (43.8)	29 (38.2)	24 (48.0)	11 (45.8)
G	143 (66.8)	74 (59.7)	27 (56.3)	47 (61.8)	26 (52.0)	13 (54.2)

a: percent

**Table III.** CTLA-4 (-308C/T) MseI RFLP in SSc.

No. of cases	Normal 107	Whole SSc 62	Diffuse 24	Limited 38	a-Scl-70+ 25	ACA+ 12
Genotype freq.						
CC	83 (77.6) <sup>a</sup>	44 (71.0)	19 (79.2)	25 (65.8)	19 (76.0)	7 (58.3)
CT	21 (19.6)	18 (29.0)	5 (20.8)	13 (34.2)	6 (24.0)	5 (41.7)
TT	3 (2.8)	0	0	0	0	0
Phenotype freq.						
C	104 (97.2)	62 (100)	24 (100)	38 (100)	25 (100)	12 (100)
T	24 (22.4)	18 (29.0)	5 (20.8)	13 (34.2)	6 (24.0)	5 (41.7)
Allele freq.						
C	187 (87.4)	106 (85.5)	43 (89.6)	63 (82.9)	44 (88.0)	19 (79.2)
T	27 (12.6)	18 (14.5)	5 (10.4)	13 (17.1)	6 (12.0)	5 (20.8)

a: percent

**Table IV.** Association of CTLA-4 (+49A/G) RFLP with autoantibody in SSc.

No. of cases	Normal 107	a-RNP +	a-SS-A -	+	-
Genotype freq.					
AA	13 (12.1) <sup>a</sup>	5 (29.4)	7 (15.6)	4 (19.0)	8 (19.5)
AG	45 (42.1)	8 (47.1)	18 (40.0)	10 (47.6)	16 (39.0)
GG	49 (45.8)	4 (23.5)	20 (44.4)	7 (33.3)	17 (41.5)
Phenotype freq.					
A	58 (54.2)	13 (76.4)	25 (55.6)	14 (66.7)	24 (58.5)
G	93 (87.8)	12 (70.6)	38 (84.4)	17 (81.0)	33 (80.5)
Allele freq.					
A	71 (33.2)	18 (52.9) <sup>b</sup>	32 (35.6)	18 (42.9)	32 (39.0)
G	143 (66.8)	16 (47.1)	58 (64.4)	24 (57.1)	50 (61.0)

a: percent b: p = 0.0337 (comparing to Normal), OR = 2.27 (1.09-4.71).

whole SSc, diffuse type, limited type, a-Scl-70 positive group and ACA positive group, but the increases were all not significant.

Genotype frequencies of the -308C/T dimorphism are shown in Table III. In all SSc groups, no significant association of any -308C/T genotypes was ob-

served. No significant difference in allele frequency between the control and each SSc group was observed either.

For examining the association of autoantibodies and CTLA-4 dimorphisms in SSc, a-RNP, a-SSA and a-SSB were studied in SSc. The association be-

tween the CTLA-4 +49A/G dimorphism and autoantibodies are shown in Table IV. The genotype +49AA increased (29.4%) and the genotype +49GG decreased (23.5%) in the a-RNP positive group, though these differences were not significant. Allele frequency of the +49A significantly increased in the a-RNP positive group [52.9% Vs 33.2% in control, p = 0.0337, OR = 2.27 (CI = 1.09 - 4.71)]. No association was observed between a-SS-A and the CTLA-4 +49A/G dimorphism, and a-SS-B failed to show an association, as well (data was not shown due to the small number of positive patients). No significant association between the -308C/T dimorphism and autoantibodies examined was observed either (data was not shown).

Associations between the CTLA-4 +49A/G and the -308C/T were studied in the control group. The +49A positively associated with the -308T (p = 1.06 x10<sup>-6</sup>) and the +49G positively associated with the -308C (p = 0.0014). HLA-DRB1 was examined in this study because it had been reported that the +49GG associated with RA carrying DRB1\*0401 (22). In this study, 30 patients in whole SSc had either the HLA-DRB1\*1502-DRB5\*0102 haplotype or DRB1\*0802 (tentatively named as SSc-susceptibility HLA-DR epitope in this paper) (48.4% vs 25.0% in normal [N = 104 (3)], p = 0.0037, OR = 2.82 [CI = 1.44 - 5.48]). In diffuse scleroderma, 21 patients were positive for the epitope [87.5%, p = 0.00000002, OR = 21.00 (CI = 5.79 - 76.18)] and 17

**Table V.** Synergistic effect of SSc-susceptibility HLA-DR epitope on CTLA-4 RFLP.

No. of cases	Whole SSc		Diffuse		a-Scl-70+		a-RNP+	
	Epitope+ <sup>a</sup> 30	Epitope- 32	Epitope+ 21	Epitope- 3	Epitope+ 17	Epitope- 8	Epitope+ 8	Epitope- 9
Genotype freq.								
AA	6 (20.0) <sup>b</sup>	6 (18.8)	5 (23.8)	0	4 (23.5)	1 (12.5)	2 (25.0)	3 (33.3)
AG	14 (46.7)	12 (37.5)	10 (47.6)	1 (33.3)	9 (52.9)	5 (62.5)	4 (50.0)	4 (44.4)
GG	10 (33.3)	14 (43.8)	6 (25.6)	2 (66.7)	4 (23.5)	2 (25.0)	2 (25.0)	2 (22.2)
Allele freq.								
A	26 (43.3)	24 (37.5)	20 (47.6)	1 (16.7)	17 (50.0)	7 (43.8)	8 (50.0)	10 (55.6)
G	34 (56.7)	40 (62.5)	22 (52.4)	5 (83.3)	17 (50.0)	9 (56.3)	8 (50.0)	8 (44.4)

a: Patients with HLADRB1\*1502-DRB5\*0102 or HLADRB1\*0802. DRB5\*0102 and DRB1\*0802 have common amino acid sequence (V<sup>38</sup>F<sup>67</sup>LED<sup>71</sup>). In this paper, we indicated the sequence tentatively as SSc epitope for discussion.

b: percent

patients (68.0%,  $p = 0.00009$ ,  $OR = 6.38$  ( $CI = 2.46 - 16.49$ )) were positive for the epitope in the a-Scl-70 positive group. These data reconfirm our previously reported results (3, 27).

In Table V, genotype frequencies and allele frequencies of the +49A/G dimorphism are shown in SSc groups with and without the epitope. Genotype frequencies of the AA (+49A/G) did not significantly increase in the epitope positive sub-groups of whole SSc (20.0%), diffuse scleroderma (23.8%) and a-Scl-70 positive (23.5%). Allele frequencies of the +49A also increased in each subgroup (43.3%, 47.6% and 50.0%, respectively), but no significant difference was observed. In a-RNP positive SSc, 8 patients (47.1%) were positive for the HLA-DR epitope. In patients with a-RNP and the DR epitope, the frequency of genotype +49AA was 25.0% and allele frequency of the +49A was 50% and these increases were not significant. There was no significant association between the -308C/T dimorphism and each SSc group positive for the epitope.

## Discussion

Though associations between CTLA-4 and connective tissue diseases including RA and SLE have been reported, these results are controversial (15, 16, 21-23) and the contribution of CTLA-4 genes to the pathogenesis of connective tissue diseases is still unclear. On the other hand, no study has been reported on the association of CTLA-4 dimorphisms and SSc. In this experiment, using Japanese SSc patients, no association of CTLA-4 dimorphisms (+49A/G and -308C/T) was observed in scleroderma, though slight increases of the +49AA genotype and the A allele were observed in SSc.

In this study, the synergistic effect of SSC-susceptibility HLA-DR epitope on CTLA-4 RFLP (3, 4) was examined, because associations of CTLA-4 with HLA-DR shared epitope have been reported in RA (22, 28). For SSc, an association with HLA-DRB and a specific amino acid sequence in the DR chain has yet to be recognized and established generally, though diffuse scleroderma and a-Scl-70 positive SSc are

strongly associated with HLA-DRB1\*1502 - DRB5\*0102 haplotype and DRB1\*0802 in Japanese (3,4). The association was recently re-confirmed in Korean SSc (29). Both DRB5\*0102 and DRB1\*0802 have a common amino acid sequence, V<sup>38</sup>F<sup>67</sup> LEDR<sup>71</sup> (3, 4). HLA-DRB1\*11, which has been associated with Caucasian SSc also has this amino acid sequence. In this report, we tentatively indicated the sequence as SSc-susceptibility HLA-DR epitope.

In Japanese SSc with the SSc-susceptibility HLA-DR epitope, no significant association of CTLA-4 dimorphisms (+49A/G and -308C/T) with whole SSc and diffuse scleroderma was observed, though frequencies of genotype +49AA and +49AG, as well as the allele frequency of the +49A had also slightly increased. In a-Scl-70 positive SSc with the epitope, CTLA-4 dimorphisms (+49A/G and -308C/T) were not associated with the SSc sub-group, though genotype frequencies of the +49AA and the +49AG, and allele frequency of the +49A had once again slightly increased. Despite the fact that no direct association was observed between the SSc-susceptibility HLA-DR epitope and a-RNP presence (3), the synergistic effect of the epitope in SSc with a-RNP was studied because the frequency of the +49A allele increased significantly in SSc with a-RNP. The frequency of the +49A in a-RNP positive SSc with the epitope was almost equal to the frequency in a-RNP positive SSc without the DR epitope (50.0% and 55.6%, respectively). The frequencies in both a-RNP positive groups did not significantly increase statistically, probably as a result of the small number of samples. In Japanese SSc, no synergistic effect of SSc-susceptibility HLA-DR epitope on CTLA-4 dimorphism was observed in the pathogenesis of SSc.

The significant association between a-RNP and the +49A allele was observed in Japanese SSc. No significant associations of a-Scl-70, ACA or a-SS-A with CTLA-4 were observed in this study. The presence of a-RNP was relatively high in limited scleroderma. It is rare in SSc that a-RNP is detected together with a-Scl-70 or ACA (30). Moreover, relatively mild lung fibrosis has been

clinically reported in scleroderma with a-RNP (31). In this experiments prevalences of lung fibrosis were 64.5%, 83.3% and 52.6% in whole SSc, diffuse scleroderma and limited scleroderma, respectively. Among patients used in this study, the frequency of lung fibrosis was 35.3% in a-RNP positive SSc and was significantly low when compared to the frequency in a-RNP negative SSc (75.6%) ( $p = 0.0021$ ,  $OR = 5.67$  ( $CI = 1.70 - 18.91$ )). Primarily in limited scleroderma, the frequency of lung fibrosis was significantly low in the a-RNP positive group in comparison with the a-RNP negative group (23.1% Vs 68.0%,  $p = 0.0156$ ,  $OR = 7.08$  ( $CI = 1.52 - 33.03$ )). In SSc without lung fibrosis, the +49AA genotype increased significantly (31.8%,  $p = 0.0456$ ,  $OR = 3.37$  ( $CI = 1.16 - 9.82$ )). In limited scleroderma without lung fibrosis, the +49AA genotype again increased significantly (33.3%,  $p = 0.0319$ ,  $OR = 3.62$  ( $CI = 1.16 - 11.29$ )). Allele frequency of the +49A in limited SSc with lung fibrosis was 30.0%, and that was relatively low in comparison with that in limited SSc without lung fibrosis (47.2%). The frequency of the +49A in SSc without lung fibrosis increased slightly, but the increase was not significant in comparison with that of the control (Table VI). Allele frequency of the -308T did not significantly increase in limited scleroderma without lung fibrosis (25.0%), possibly as a result of the positive association between the +49A allele and the -308T allele.

A negative association between a-RNP and lung fibrosis of SSc was not established in general, yet. In this study, a weak but significant association of the +49A allele with a-RNP was observed. Additionally our data suggested that the +49AA genotype could be associated with limited SSc without lung fibrosis, and the +49A allele would contribute protectively to lung fibrosis in limited scleroderma. It is, however, difficult to clarify the role of the +49A allele in lung fibrosis in SSc because of the relatively small number of cases available for clinical analysis and for discussing the significance. In this paper only the possibility was shown

**Table VI.** Association of CTLA-4 (+49A/G) RFLP with lung lesion in SSc.

	N	Lung fibrosis	
		+	-
Whole SSc	62	40	22
AA		5 (12.5)	7 (31.8) <sup>b</sup>
AG		21 (52.5)	5 (22.7)
GG		14 (35.0)	10 (45.5)
allele A <sup>a</sup>		31 (38.8)	19 (43.2)
G		49 (61.3)	25 (56.8)
Diffuse SSc	24	20	4
AA		4 (20.0)	1 (25.0)
AG		11 (55.0)	0
GG		5 (25.0)	3 (75.0)
allele A		19 (47.5)	2 (25.0)
G		21 (52.5)	6 (75.0)
Limited SSc	38	20	18
AA		1 (5.0)	6 (33.3) <sup>c</sup>
AG		10 (50.0)	5 (27.8)
GG		9 (45.0)	7 (38.9)
allele A		12 (30.0)	17 (47.2)
G		28 (70.0)	19 (52.8)

Frequencies were compared with those in normal control.

a: allele frequency; b:  $p = 0.0456$ , OR = 3.37 (CI = 1.16 - 9.82); c:  $p = 0.0319$ , OR = 3.62 (CI = 1.16 - 11.29).

and discussed.

Our observations showed no association of CTLA-4 dimorphisms (+49A/G and -308C/T) with SSc. The CTLA-4 dimorphisms were not associated with disease types of SSc. In a-RNP positive patients, the +49A allele increased significantly and there was a possibility that the allele could contribute protectively to lung fibrosis. Further information on the genetical analysis of CTLA-4 will be necessary in order to clarify the contribution of CTLA-4 to the clinical feature of SSc.

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