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Association of Leukocyte Immunoglobulin-like Receptor A3 (LILRA3) Deletion Polymorphism with Anti-topoisomerase I Antibody Positive Systemic Sclerosis: A Case-Control Association Study.

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Abstract:	The leukocyte immunoglobulin-like receptors (LILRs) are a family of receptors with immunoregulatory functions. LILRA3 has a 6.7-kb deletion polymorphism, whose frequency is especially high in the Northeast Asian populations. Previous studies reported association of the deletion allele with multiple sclerosis, Sjögren's syndrome (SS) and B-non-Hodgkin's lymphoma in Europe, while homozygous non-deletion genotype has been associated with rheumatoid arthritis, systemic lupus erythematosus and SS in China. Here we examined association of LILRA3 deletion with systemic sclerosis (SSc) in 378 Japanese patients and 867 healthy controls. Among the patients, 88 were positive for anti-topoisomerase I antibody (ATA+), and 172 were positive for anti-centromere antibody (ACA+). Although significant association with overall SSc was not detected, the LILRA3 deletion allele was significantly increased in ATA+ SSc when compared with healthy controls (P=0.012, odds ratio [OR] 1.68, 95% confidence interval 1.12-2.52 under the allele model). In addition, a case-case analysis revealed that the LILRA3 deletion allele was significantly increased in ATA+ACA- SSc when compared with ATA-ACA+ SSc (P=0.020, OR 1.77, 95%CI 1.09-2.88 under the allele model). Although further replication studies are necessary, this study suggested association of LILRA3 deletion with susceptibility to ATA+SSc, supporting the emerging role of LILRA3 in the genetics of multiple autoimmune diseases.
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Competing Interests You are responsible for recognizing and disclosing on behalf of all authors any competing interest that could be perceived to bias their work, acknowledging all financial support and any other relevant financial or non-financial competing interests. Do any authors of this manuscript have competing interests (as described in the	I have read the journal's policy and the authors of this manuscript have the following competing interests: AK received a research grant from SENSHIN Medical Research Foundation, supported by an endowment from Mitsubishi Tanabe Pharma Corporation and a research grant from the Takeda Science Foundation supported by an endowment from Takeda Pharmaceutical Company. KT is a consultant for Actelion Ltd. NT received a research grant from SENSHIN Medical Research Foundation, supported by an endowment from Mitsubishi Tanabe Pharma Corporation, received speaker's honoraria from Eisai Co., Ltd., Daiichi Sankyo Co., Ltd. and Asahi Kasei Corporation, and payment for a manuscript from Torii Pharmaceutical Co., Ltd. None of the above is directly relevant to this study. The other authors declare no financial or commercial conflict of interest.

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Additional data availability information:	

Title: Association of Leukocyte Immunoglobulin-like Receptor A3 (*LILRA3*) Deletion Polymorphism with Anti-topoisomerase I Antibody Positive Systemic Sclerosis: A Case-Control Association Study.

Short Title: *LILRA3* Deletion in ATA-positive Systemic Sclerosis

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ABSTRACT

The leukocyte immunoglobulin-like receptors (LILRs) are a family of receptors with immunoregulatory functions. *LILRA3* has a 6.7-kb deletion polymorphism, whose frequency is especially high in the Northeast Asian populations. Previous studies reported association of the deletion allele with multiple sclerosis, Sjögren's syndrome (SS) and B-non-Hodgkin's lymphoma in Europe, while homozygous non-deletion genotype has been associated with rheumatoid arthritis, systemic lupus erythematosus and SS in China. Here we examined association of *LILRA3* deletion with systemic sclerosis (SSc) in 378 Japanese patients and 867 healthy controls. Among the patients, 88 were positive for anti-topoisomerase I antibody (ATA+), and 172 were positive for anti-centromere antibody (ACA+). Although significant association with overall SSc was not detected, the *LILRA3* deletion allele was significantly increased in ATA+ SSc when compared with healthy controls (P=0.012, odds ratio [OR] 1.68, 95% confidence interval 1.12-2.52 under the allele model). In addition, a case-case analysis revealed that the *LILRA3* deletion allele was significantly increased in ATA+ACA- SSc when compared with ATA-ACA+ SSc (P=0.020, OR 1.77, 95%CI 1.09-2.88 under the allele model). Although further replication studies are necessary, this study suggested association of *LILRA3* deletion with susceptibility to ATA+SSc, supporting the emerging role of *LILRA3* in the genetics of multiple autoimmune diseases.

INTRODUCTION

The leukocyte immunoglobulin (Ig) - like receptors (LILRs, also referred to as immunoglobulin-like transcripts [ILTs] and LIRs) are encoded by a gene family located in leukocyte receptor complex (LRC) region at chromosome 19q13.4, along with other Ig-like receptor groups such as killer cell Ig-like receptor (*KIR*), leukocyte-associated Ig-like receptor (*LAIR*) and Fcα receptor (*FCAR*) genes. *LILR*s constitute of 13 genes including two pseudogenes (*LILRP1* and *LILRP2*) [1,2].

LILRs are expressed mainly in immune cells as transmembrane receptors. There are two groups in the LILR family which transmit activation and inhibitory signals, respectively. The activating LILRs (LILRA1, 2, 4-6) are associated with Fc receptor γ (FcRγ) chain that bears immunoreceptor tyrosine-based activation motif (ITAM). On the other hand, the inhibitory LILRs (LILRB1-5) have long cytoplasmic regions that contain immunoreceptor tyrosine-based inhibitory motif (ITIM) [1-3].

LILRA3 (ILT6, LIR-4) lacks both transmembrane and intracellular domains, and exists in human serum as secreted protein. LILRA3 shows high amino acid sequence homology to the extracellular domains of LILRB1, LILRB2 and LILRA1, and the ligands of LILRA3 have been reported to include HLA-A, -C, -G, and β_2 microglobulin-free HLA-class I heavy chain [4,5]. Of particular interest, *LILRA3* has a 6.7-kb deletion polymorphism which lacks most of the coding region [6]. Moreover, the deletion allele frequency is especially high in the Northeast Asian populations (e.g. 71.0% in the Japanese population) [7] in contrast to the Caucasian populations (e.g. 18.5% in the German population) [8]. Positive selection has been reported to play a role [9], although the nature of the selection pressure remains unclear.

Several lines of recent studies disclosed a role of *LILR* family genes in the genetics of autoimmune diseases [10] and infections [11]. With respect to *LILRA3*, increased frequency of the deletion allele has been reported in multiple sclerosis (MS) [8], Sjögren's syndrome (SS) [12] and B-non Hodgkin’s lymphoma (B-NHL) [13] in the German populations. In contrast, recent studies from China reported increased frequency of homozygous non-deletion genotype in rheumatoid arthritis (RA) [14],

systemic lupus erythematosus (SLE) and SS [15]. Thus, the direction of association of *LILRA3* deletion with autoimmune diseases remains somewhat controversial, and more studies on multiple populations are necessary.

Systemic sclerosis (SSc) is an autoimmune rheumatic disease characterized by fibrosis of skin and visceral organs, vascular damage and autoimmunity. Candidate gene studies as well as genome-wide association studies are beginning to identify some susceptibility genes; however, many more remain to be detected [16,17]. SSc is classified into diffuse cutaneous SSc (dcSSc) and limited cutaneous SSc (lcSSc) depending on the extent of skin involvement [18]. In addition, SSc can be subdivided according to the pattern of autoantibodies, which are generally non-overlapping and associated with clinical phenotypes [16]. A number of previous studies showed that genetic background may be substantially different between dcSSc and lcSSc, as well as between anti-topoisomerase I antibody positive (ATA+) and anti-centromere antibody positive (ACA+) SSc [16,19]. Our group reported that *IRF5* [20] and *UBE2L3* [21] are preferentially associated with dcSSc and ATA+ SSc, while *STAT4* [22] was predominantly associated with lcSSc and ACA+ SSc, in the Japanese. *BLK* was associated with overall SSc, but the strongest effect was observed in ACA+ SSc [23]. In the Caucasian populations, a large-scale genome-wide association study detected several new associations with SSc subsets, and also confirmed preferential association of *STAT4* and *BLK* with the ACA+ subset [19]. Thus, genetic association with SSc should be examined separately for the subsets of SSc.

To date, association studies between *LILRA3* deletion and SSc have not been reported. In view of the recent studies suggesting its role in multiple autoimmune diseases, this study examined whether *LILRA3* deletion polymorphism is associated with susceptibility to SSc and its subsets in a Japanese population.

SUBJECTS AND METHODS

Subjects

Case-control and case-case association studies were performed on 378 Japanese patients with SSc and 867 healthy Japanese controls, recruited at the universities and rheumatology centers participated in this study. All patients fulfilled the American College of Rheumatology 1980 criteria [24]. 127 were classified into dcSSc, while 231 into lcSSc, according to the classification by LeRoy et al [18]. 88 were positive for ATA, 172 for ACA, and eight were positive for both ATA and ACA. 151 patients were classified as having interstitial lung disease (ILD) based on high resolution CT.

This study was reviewed and approved by the ethics committees of University of Tsukuba, Kanazawa University, National Hospital Organization Sagamihara Hospital, Nagoya Medical Center, Himeji Medical Center, Morioka Hospital, Kyushu Medical Center, Nagasaki Medical Center, Tokyo Metropolitan Tama Medical Center, Komagome Hospital, Yokohama Minami Kyosai Hospital, Kitasato University and the University of Tokyo. Written informed consent was provided by all subjects. This study was conducted in accordance with the Declaration of Helsinki.

Genotyping

The *LILRA3* deletion was genotyped by PCR-sequence specific primers (SSP) with genomic DNA from patients and healthy controls as previously described [7] (Figure 1). The sequences of the primers are shown in Table 1. The primer-1f and -1r were placed inside the deletion sequence, and amplify 166bp fragment only from the non-deletion allele. In contrast, the primer-del-f and -del-r are located on each side of the deletion site, and amplify 237bp fragment only from the deletion allele. These four primers were put into the reaction mixture together, and multiplex PCR was performed. AmpliTaq Gold® 360 Master Mix (Applied Biosystems, Foster City, CA) 5 μl was used in 10 μl reaction mixture containing 0.1 μM of each primer.

Because *LILR* genes are highly homologous, we employed strict PCR conditions to achieve specific amplification of *LILRA3*. The PCR conditions consisted of initial

denaturation at 95°C for 10 min, followed by 36 cycles of denaturation at 95°C for 30s, annealing at 60°C for 20s, and extension at 72°C for 30s. The PCR products were detected by 10.0% acrylamide gel electrophoresis and staining using SYBR® Gold nucleic acid gel stain (Life technologies, OR).

The genotyping results were confirmed by Sanger sequencing. The primer-1f and -1r were used for sequencing of the non-deletion allele products. Because primer-del-r was designed closely to the deletion site for sequencing purpose, we amplified the deletion allele using primer-del-f and -del-r2 by PCR, and performed Sanger sequencing using primer-del-seq (Table 1).

Statistical analysis

Association analysis was performed by chi-square test using 2x2 contingency tables. P values less than 0.05 were considered significant. When the 2x2 table contains the frequency equal to or less than 5, Fisher's exact test was employed. Adjustment for gender difference between the patients and the controls was performed using logistic regression analysis. Because only one polymorphic site was examined, and each of the patients' subgroups was not independent, correction for multiple testing was not performed. Power calculation using the PS (Power and Sample Size Calculation) program (http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize#PS:_Power_and_Sample_Size_Calculation) [25] is shown in Table 2

RESULTS

Table 3 shows the results of *LILRA3* deletion genotyping and association test in 387 Japanese SSc patients and 867 healthy controls. None of the genotype frequencies of the patients, patients' subgroups and healthy controls were significantly departed from Hardy-Weinberg equilibrium (P>0.25).

Although the deletion allele showed a tendency toward increase in overall SSc, the difference did not reach statistical significance.

When the subsets of SSc were compared with healthy controls, the deletion allele was significantly increased in SSc patients positive for ATA (ATA+ SSc) (P=0.012, odds ratio [OR] 1.68, 95% confidence interval [CI] 1.12-2.52 under the allele model, P=0.013, OR 1.81, 95% CI 1.14-2.89 under the recessive model for the deletion allele). This association remained significant after adjusting for the gender difference between the patients and the controls, using logistic regression analysis (P=0.018, OR 1.64, 95%CI 1.09-2.46 under the additive model, P=0.018, OR 1.77, 95%CI 1.10-2.85 under the recessive model).

On the other hand, significant association was not observed in SSc patients positive for ACA (ACA+ SSc). A tendency toward increase in the deletion allele was observed in dcSSc group as well as in the patients accompanied by interstitial lung diseases (ILD), but the association did not reach statistical significance.

We also performed a case-case analysis between ATA+ACA- SSc and ATA-ACA+ SSc by excluding 8 patients who were positive for both antibodies (Table 4). This analysis confirmed significant increase of the deletion allele in ATA+ACA- SSc (P=0.020, OR 1.77 under the allele model, P=0.028, OR 1.89 under the recessive model).

DISCUSSION

The present study suggested that the *LILRA3* deletion might be a genetic risk factor for ATA+ SSc in a Japanese population. Furthermore, the *LILRA3* deletion allele also showed a tendency toward increase in dcSSc, and SSc accompanied by ILD. Because these three phenotypes substantially overlap and define the severe subset of SSc, our results imply that *LILRA3* deletion may be associated with the risk of the severe subphenotype of SSc.

It is interesting to note that the proportion of ATA+ SSc and dcSSc is higher in Asian populations than in other populations [26]. Thus far, population differences in the HLA-class II allele frequencies were considered, at least in part, to account for such a population difference in the disease phenotype [27]. This study suggested that, in view of the striking population difference in the allele frequency between East Asian and other populations, it is possible that *LILRA3* may also play a role in the population difference in ATA+ SSc.

The *LILRA3* deletion allele has previously been associated with risk to MS, SS and B-NHL in the German population [8,12,13]. However, in the recent studies from China, homozygous non-deletion genotype was reported to be associated with the risk to RA [14], SS and SLE [15]. In the shared susceptibility genes to autoimmune diseases, the risk allele in each gene is in many cases the same among the diseases [28]; however, in some genes, opposite directions of association were reported according to diseases [29]. For example, in *SPP1* gene coding for osteopontin, the risk alleles at rs11439060 and rs9138 were found to be opposite between SLE and anti-citrullinated protein antibody negative RA [30]. In the case of *LILRA3* deletion, however, opposite directions were reported in the same disease, SS, in the German and Chinese populations [12,15]. Our observations on ATA+ SSc is compatible with the risk allele to various autoimmune diseases reported in the German population. In order to gain more clear view on this issue, more studies in multiple populations are required.

A recent study reported that LILRA3 protein bound to monocytes and B cells, and induced proliferation of CD8+ T cells and NK cells in mixed lymphocyte reaction via

modulating the function of monocytes, or by inducing secretion of IL-6, IL-8, IL-1β and IL-10 [13]. On the other hand, HLA-G, one of the possible ligands of LILRA3, has been shown to have immunosuppressive functions and to play a role in SLE and RA [31]. Interestingly, expression of HLA-G has been shown in the skin of SSc [32]. It might be possible to speculate that HLA-G is somehow involved in the pathogenesis of SSc, and LILRA3, a soluble receptor, may have an inhibitory role. Thus, the deletion of *LILRA3* could result in the loss of inhibition, and lead to the development of SSc. All of these hypotheses remain speculative and await future studies.

This study suffers from some limitations. Due to the low prevalence, the sample size, especially that of ATA+ SSc was limited. In view of the striking difference of *LILRA3* deletion frequency between the European and Asian populations, we considered that trans-ethnic replication study may not be an ideal approach. Lack of significant association in dcSSc and SSc accompanied by ILD might be due to low detection power (Table 2). Thus, future independent studies are necessary to validate our observations.

In conclusion, this study demonstrated the first evidence for the association of *LILRA3* deletion with the genetic susceptibility to ATA+ SSc. This finding supported the emerging role of LILRs in multiple conditions.

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Conflict of Interest Statement:

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Figure Legend

Figure 1. Genomic organization of *LILRA3* deletion polymorphism and genotyping system

(A) *LILRA3* consists of seven exons in the non-deletion allele (+). In the deletion allele (-), 6.7 kb fragment encompassing exon 1 through exon 6 as well as the flanking regions is deleted. To genotype this indel polymorphism, two sets of primers (arrows) were designed. Primer-1f and -1r were placed inside the deletion segment and amplify 166 bp fragment only from the non-deletion allele. Primer-del-f and –del-r were placed on each side of the deletion site, and amplify 237 bp fragment only from the deletion allele. To determine the genotype, both primer sets were used in a single PCR reaction, and multiplex PCR was performed.

(B) In the homozygotes of the non-deletion allele (+/+), only the 166 bp band was detected, and in the homozygotes of the deletion allele (-/-), only the 237 bp band. The heterozygotes exhibited both 166 bp and 237 bp bands.

Table 1. Sequence of primers used in this study.

primer	
-1f	5'-GAGAACACATCAATCATCCAACG-3'
-1r	5'-TCCTTTGCAGCTTGTTGTCACA-3'
-del-f	5'-CTCGATCTGCCACTGACAC-3'
-del-r	5'-ACAGCAGATTCTAAAACAGTG-3'
-del-r2	5'-CGCCTGTAGTCCCAGCTAC-3'
-del-seq	5'-TTTTGAGATGGAGTCTCACAGT-3'

Table 2. Power calculation under the allele model.

	Odds ratio		
	1.1	1.3	1.5
all SSc	0.165	0.777	0.989
dcSSc	0.101	0.438	0.798
lcSSc	0.131	0.626	0.944
ATA+SSc	0.088	0.341	0.668
ACA+SSc	0.115	0.531	0.885
ILD	0.109	0.490	0.850

Power calculation based on the *LILRA3* deletion allele frequency and sample size of this study was carried out using the PS (Power and Sample Size Calculation) program.

Table 3. Case-control association study between *LILRA3* deletion polymorphism and SSc.

	n	genotype frequency			del allele frequency	allele		recessive		dominant	
		-/- (%)	+/- (%)	+/+ (%)		P	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)
all SSc	378	230(60.9)	128(33.9)	20(5.3)	77.8%	0.067	1.21 (0.99-1.48)	0.078	1.25 (0.98-1.60)	0.31	1.31 (0.78-2.20)
dcSSc	127	80(63.0)	42(33.1)	5(3.9)	79.5%	0.074	1.34 (0.97-1.85)	0.11	1.37 (0.93-2.00)	0.33*	1.78 (0.71-4.47)
lcSSc	231	141(61.0)	75(32.5)	15(6.5)	77.3%	0.20	1.17 (0.92-1.50)	0.13	1.26 (0.93-1.69)	0.89	1.05 (0.58-1.89)
ATA+	88	61(69.3)	24(27.3)	3(3.4)	83.0%	0.012	1.68 (1.12-2.52)	0.013	1.81 (1.14-2.89)	0.26*	2.07 (0.65-6.58)
ACA+	172	97(56.4)	62(36.0)	13(7.6)	74.4%	0.97	1.00 (0.77-1.31)	0.83	1.04 (0.75-1.44)	0.72	0.89 (0.48-1.67)
ILD	151	92(60.9)	54(35.8)	5(3.3)	78.8%	0.098	1.28 (0.96-1.73)	0.21	1.25 (0.88-1.78)	0.14*	2.13 (0.86-5.29)
Healthy controls	867	481(55.5)	327(37.7)	59(6.8)	74.3%	ref		ref		ref	

－: deletion allele, +: non-deletion allele, OR: odds ratio, 95%CI: 95% confidence interval, dcSSc: diffuse cutaneous SSc, lcSSc: limited cutaneous SSc, ATA+: anti-topoisomerase I antibody positive SSc, ACA+: anti-centromere antibody positive SSc, ILD: SSc with interstitial lung disease, allele model: comparison of – vs +, recessive model: comparison of -/- vs (+/- or +/+), dominant model: comparison of (-/- or +/-) vs +/+.

*Fisher’s exact test.

Table 4. Case-case association study between ATA+ACA- SSc and ATA-ACA+ SSc.

	n	genotype frequency			del allele frequency	allele		recessive		dominant	
		-/-(%)	+/-(%)	+/+(%)		P	OR (95%CI)	P	OR (95%CI)	P	OR (95%CI)
ATA+ACA-	80	57(71.3)	20(25.0)	3(3.8)	83.8%	0.020	1.77 (1.09-2.88)	0.028	1.89 (1.07-3.35)	0.27*	2.21 (0.63-7.78)
ATA-ACA+	164	93(56.7)	58(35.4)	13(7.9)	74.4%	ref		ref		ref	

－: deletion allele, +: non-deletion allele, OR: odds ratio, 95%CI: 95% confidence interval,

ATA+ACA-: anti-topoisomerase I antibody positive and anti-centromere antibody negative SSc,

ATA-ACA+: anti-topoisomerase I antibody negative and anti-centromere antibody positive SSc,

allele model: comparison of – vs +, recessive model: comparison of -/- vs (+/- or +/+), dominant model: comparison of (-/- or +/-) vs +/+.

*Fisher's exact test.

Figure
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