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Killer cell immunoglobulin-like receptor (KIR) genes in systemic sclerosis

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Summary

A previous study has suggested that the combination *KIR2DS2*$^+$/*KIR2DL2*$^-$ was related to increased risk for systemic sclerosis (SSc), while others have failed to reproduce this finding. Our objective was to study this matter further and test the association of other *KIR* genes with SSc. One hundred and ten SSc patients and 115 healthy bone marrow donors were enrolled in a case–control study. Blood was collected for DNA extraction; typing of 15 *KIR* genes and human leucocyte antigen-C (HLA-C) was made by polymerase chain reaction with sequence specific primers (PCR–SSP), followed by electrophoresis on agarose gel. Patients underwent clinical evaluation, serology, Doppler echocardiography and chest high-resolution computed tomography. The frequency of the inhibitory *KIR2DL2* was significantly lower in patients [29·1% *versus* 65·2% in controls, $P < 0{\cdot}0001$; odds ratio (OR) = 0·22, 95% confidence interval 0·12–0·40]. When combinations of activating and inhibitory *KIR* genes were analysed, the presence of *KIR2DS2* in the absence of *KIR2DL2* (*KIR2DS2*$^+$/*KIR2DL2*$^-$) was more frequent in patients than in controls (25·5% *versus* 1·7%, respectively; $P < 0{\cdot}0001$; OR = 19·29, 4·24–122·26). However, the presence of both *KIR2DS2* and *KIR2DL2* (*KIR2DS2*$^+$/*KIR2DL2*$^+$) was more frequent in controls (57·4%) than in patients (28·2%, $P < 0{\cdot}0001$), suggesting a preponderant protective effect of *KIR2DL2* over *KIR2DS2*. Stratification for HLA-C1 status did not change these results. No statistically significant associations were found between *KIR* phenotypes and clinical and laboratory features of SSc. Our results suggest a protective role of *KIR2DL2*$^+$ phenotype and confirmed the association of the combination *KIR2DS2*$^+$/*KIR2DL2*$^-$ with increased risk for SSc.

Keywords: case–control studies, genetic predisposition to disease, KIR receptors, KIR2DL2 receptors, systemic scleroderma

Introduction

Systemic sclerosis (SSc) is a diffuse connective tissue disease characterized by autoimmunity, vascular dysfunction and variable degrees of fibrosis in the skin and internal organs. Its pathogenesis is not well known, but evidence suggests an inappropriate activation of the immune system triggered by some environmental stimuli in individuals with a genetic background of susceptibility [1].

Activation of oligoclonal T cells has been implicated in the pathogenesis of systemic sclerosis, suggesting an antigen-driven immune response in this disease [2]. The activation of T cells is mediated through T cell receptors (TCR), and this activation can be modulated by killer immunoglobulin-like receptors (KIR) [3,4]. KIR are members of the immunoglobulin superfamily and are expressed on natural killer (NK) cells and subsets of T cells. Depending on their structure, they can generate activating or inhibitory signals [5]. Inhibitory KIR molecules bind to target cell major histocompatibility complex (MHC) class I molecules and prevent the attack of NK cells on normal cells [5]. The capacity to attack self cells that lack expression of MHC class I molecules is known as 'missing self recognition' [6,7]. The missing-self hypothesis has been supported by several independent findings demonstrating that allotypic MHC products actually protect cells from lysis by NK lymphocytes, apparently by delivering negative signals that inhibit NK cell cytotoxic function [7]. On the other hand, when an activating KIR

binds to its ligand, activating signals are generated leading to the kill of the target cells. Besides the modulation of TCR-mediated activation of T cells, KIR expression may affect the role of NK cells in autoimmune diseases, where these cells may exert a pathogenic function through inappropriate activation or suppression function through lysis of dendritic cells or activated T cells [5]. Therefore, genes that control KIR expression may possibly influence normal and pathological immune responses.

To date, 17 *KIR* genes and pseudogenes have been described on human chromosome 19q13.4 (~0·7 Mb) [8]. Eight genes that encode KIR receptors are inhibitory (*2DL1*, *2DL2*, *2DL3*, *2DL5A*, *2DL5B 3DL1*, *3DL2* and *3DL3*), seven are activating (*2DL4*, *2DS1*, *2DS2*, *2DS3*, *2DS4*, *2DS5*, *2DS5* and *3DS1*) and two are pseudogenes (*2DP1* and *3DP1*). Of these, four *KIR* genes are always present: *3DL3*, *3DP1*, *2DL4* and *3DL2*. They are considered framework genes [9].

A previous study by Momot *et al.* [10] suggested that the presence of $KIR2DS2^{+}$, in the absence of $KIR2DL2^{-}$, is associated with SSc. In contrast, Pellet *et al.* [11] found association of the disease with the presence of *KIR2DS1* and the absence of *KIR2DS2*. Given these contradictory results, we designed a study to investigate further the association of *KIR* genes with systemic sclerosis.

Methods

Patients and controls

One hundred and ten patients with systemic sclerosis were evaluated prospectively in the out-patient clinic of the Service of Rheumatology at the Hospital de Clínicas de Porto Alegre. All patients met the American College of Rheumatology (ACR) criteria for SSc [12] or the criteria suggested by LeRoy and Medsger for diagnosis of early forms of SSc [13]. All patients were Brazilian (92 women and 18 men; 81·8% European descendents and 18·2% African descendents) and most of them lived in the metropolitan area of Porto Alegre/RS. There were neither individuals of Asiatic origin nor Amerindians among the patients. Patients with overlapping syndromes were excluded. However, patients with definite diagnosis of SSc (according to the ACR criteria) who presented with secondary inflammatory myopathy or Sjögren's syndrome were not excluded from the analysis.

Controls were 115 voluntary healthy bone marrow donors recruited at the blood bank of the Service of Immunology at the Hospital de Clínicas de Porto Alegre, most of them resident in the urban area of Porto Alegre/RS (83 women and 32 men; 86·1% European descendents and 13·9% African descendents). Individuals presenting chronic or acute diseases were excluded from the sample, as well as those presenting family history of genetic diseases (X-linked, autosomal or chromosomal abnormalities). Amerindians and subjects with Asiatic origin were not included.

Clinical evaluation

All patients were interviewed and examined according to an extensive questionnaire directed to the evaluation of end-organ damage [14]. Disease subtype was classified as follows: diffuse cutaneous SSc (truncal and acral skin tautness), limited cutaneous SSc (skin tautness restricted to extremities and/or face) and limited SSc (sine scleroderma) [13,15]. Clinical characteristics of the disease were observed and recorded as described previously [14]. Blood samples were collected for serology [anti-nuclear antibodies (ANA), anti-centromere and anti-topoisomerase I antibodies] and DNA extraction. Pulmonary high-resolution computed tomography (HRCT) was performed in most patients. Doppler echocardiography was used to estimate the pulmonary systolic arterial pressure (PSAP), and patients with PSAP $\geq$ 40 mmHg were considered to have pulmonary arterial hypertension. This study was approved by the Research Ethics Board of Hospital de Clínicas de Porto Alegre (IRB0000921). All patients and controls signed a written informed consent before participating in this study.

KIR typing

DNA was extracted from blood buffy coat using a modified salting-out technique, as described by Miller SA *et al.* [16]. Fifteen *KIR* genes (*2DS1*, *2DS2*, *2DS3*, *2DS4*, *2DS5*, *3DS1*, *2DL1*, *2DL2*, *2DL3*, *2DL4*, *2DL5*, *3DL1*, *3DL2*, *3DL3* and *2DP1*) were typed in patients and controls using a polymerase chain reaction with sequence specific primers (PCR–SSP) method, as described by Gomez-Lozano *et al.* [17]. For the PCR, 10 ng DNA, 50 mM $MgCl_2$, 1 µl PCR buffer, 25 mM deoxyribonucleoside triphosphates (dNTPs), 500 nM primers, 100 nm internal control and 2·5 units of *Taq* polymerase were mixed in a total volume of 10 µl [internal control primers amplify a 796 base pairs (bp) fragment of the third intron of human leucocyte antigen (HLA) DRB1]. PCR products were amplified by the GeneAmp PCR system 9600 (Perkin-Elmer, Norwalk, CA, USA), with denaturation for 3 min at 94°C, followed by four cycles of 15 s at 94°C, 15 s at 65°C, 15 s at 72°C; 21 cycles of 15 s at 94°C, 15 s at 60°C, 30 s at 72°C; five cycles of 15 s at 94°C, 1 min at 55°C, 2 min at 72°C; and a final elongation step at 72°C for 7 min. The PCR products were analysed on 1% agarose gel after electrophoresis.

HLA-C typing

HLA-C analysis was performed using PCR–SSP, as described [18]. The results of HLA-C typing were separated into two groups: HLA-C group 1 (C1), consisting of HLA-C 01, 03, 07 (01–06), 08, 12 (02, 03, 06), 14, 16 (01, 03, 04) and HLA-C group 2 (C2) consisting of HLA-C 02, 04, 05, 06, 0707, 12 (04, 05), 15, 1602, 17, 18 [19]. HLA-C group 1 (C1) mol-

Table 1. Characteristics of patients and controls.*

	Patients ($n = 110$)	Controls ($n = 115$)	P-value**
Female sex	92 (83·6)	83 (72·2)	0·056
Caucasians	90 (81·8)	99 (86·1)	0·489
Age (years) – mean (s.d.)	50·3 (14·6)	39·8 (16·7)	<0·001
Disease subtype			
Diffuse	29 (26·4)		
Limited	67 (60·9)		
Sine-scleroderma	14 (12·7)		
Fulfillment of ACR criteria	89 (80·9)		
Anti-nuclear antibodies†	95/107 (88·8)		
Anti-centromere antibodies†	37/107 (34·6)		
Anti-topoisomerase I antibodies†	26/108 (24·1)		
Interstitial lung disease on HRCT†	65/105 (61·9)		
Pulmonary arterial hypertension†	16/102 (15·7)		

*Values are number (percentage), except when indicated otherwise. **Yates' corrected χ^2 or Student's t-test. †Data not available for all patients; values represent the number of patients with the indicated abnormalities over the number of patients that were examined, with percentages given in parentheses. ACR, American College of Rheumatology; HRCT, high-resolution computed tomography; s.d., standard deviation.

ecules bind to KIR2DS2, KIR2DL2 and KIR2DL3, while group 2 (C2) molecules bind to KIR2DS1 and KIR2DL1 [20].

Statistical analysis

Data were analysed using EPI-INFO version 6·0 and SPSS version 16·0 software. The carrier frequencies (CF) were compared using Yates' corrected χ^2 or Fisher's exact test. Student's t-test and Mann–Whitney test were used to perform between-group comparisons in which the dependent variables were parametric and non-parametric, respectively. Holm's procedure for adjustment of the P-values for multiple comparisons was applied (with the aid of the WINPEPI software version 9·4) and ARLEQUIN software (version 3·01) was used to determine linkage disequilibrium (LD) [21]. The crude and Mantel–Haenszel (M–H; for stratified analysis) odds ratios (OR), along with 95% confidence intervals (95% CI), were calculated for alleles or combinations whose frequencies distributions were significantly different between patients and controls. Chi-square for evaluation of interactions was also performed. P-values less than or equal to 0·05 were considered statistically significant.

Results

The clinical and demographic features of patients and controls are shown in Table 1. There was no significant difference in the frequencies of European descendants between the study groups, but patients had a higher mean age and tended towards a higher prevalence of female sex. HLA-C1 was positive in 80 (72·7%) patients and 87 (75·7%) controls ($P = 0·727$), and HLA-C2 was present in 67 (60·9%) patients and 73 (63·5%) controls ($P = 0·795$).

Distribution of the *KIR* genes among patients and controls is compared in Table 2. The frequencies of the *KIR* genes in our control group were similar to other studies reported for Brazilian populations [22,23]. The proportion of controls with inhibitory KIR2DL2 receptors was significantly higher than that of patients with SSc (crude OR: 0·22, 95% CI: 0·12–0·40, adjusted $P < 0·0001$; M–H OR, stratified for race and sex: 0·23, 95% CI: 0·13–0·41, adjusted $P < 0·0001$). Including only patients fulfilling the ACR criteria in the analysis, the results are very similar (crude OR: 0·21, 95% CI: 0·11–0·40, adjusted $P < 0·0001$; M–H OR: 0·22, 95% CI: 0·12–0·40, adjusted $P < 0·0001$). There was a

Table 2. Frequencies of killer immunoglobulin-like receptor (*KIR*) genes in systemic sclerosis ($n = 110$) and healthy controls ($n = 115$).

KIR gene	Systemic sclerosis CF (%)	Healthy controls CF (%)	P-value*
2DL1	107 (97·3)	112 (97·4)	1·0
2DL2	32 (29·1)	75 (65·2)	<0·0001
2DL3	91 (82·7)	98 (85·2)	1·0
2DL4	108 (98·2)	115 (100·0)	1·0
2DS2	59 (53·6)	68 (59·1)	1·0
2DS3	33 (30·0)	39 (33·9)	1·0
2DS5	15 (13·6)	33 (28·7)	0·123
2DS1	22 (20·0)	44 (38·3)	0·059
3DL1	106 (96·4)	110 (95·7)	1·0
3DL2	108 (98·2)	113 (98·3)	1·0
3DS1	42 (38·2)	48 (41·7)	1·0
3DL3	107 (97·3)	113 (98·3)	1·0
2DL5	58 (52·7)	60 (52·2)	1·0
2DP1	109 (99·1)	112 (97·4)	1·0
2DS4	105 (95·4)	110 (95·7)	1·0

*Yates' corrected χ^2 or Fisher's exact test, after adjustment for multiple comparisons (15 tests). CF, carrier frequencies.

Table 3. Combinations of killer immunoglobulin-like receptor (*KIR*) genes in systemic sclerosis ($n = 110$) and healthy controls ($n = 115$).

KIR combinations	Systemic sclerosis CF (%)	Healthy controls CF (%)	*P*-value*
$2DS1^+/2DL1^-$	2 (1·8)	3 (2·6)	1·0
$2DS2^+/2DL2^-$	28 (25·5)	2 (1·7)	<0·0001
$2DS3^+/2DL3^-$	10 (9·1)	10 (8·7)	1·0
$3DS1^+/3DL1^-$	4 (3·6)	5 (4·3)	1·0
$2DS2^+/2DL2^+$	31 (28·2)	66 (57·4)	0·0001
$2DS2^-/2DL2^+$	1 (0·9)	9 (7·8)	0·115
$2DS1^+/2DS2^+$	15 (13·6)	32 (27·8)	0·099
$2DS2^-/2DL2^-$	50 (45·5)	38 (33·0)	0·383
$2DS1^+/2DS2^-$	7 (6·4)	12 (10·4)	1·0

*Yates' corrected χ^2 or Fisher's exact test, after adjustment for multiple comparisons (nine tests). CF, carrier frequencies.

statistical trend (adjusted $P = 0{\cdot}059$) for lower prevalence of *KIR2DS1* in patients. There was no significant difference in the frequencies of the other *KIR* genes.

Analysing the combinations of *KIR* genes (Table 3), an association of $KIR2DS2^+/KIR2DL2^-$ with systemic sclerosis was observed (crude OR: 19·29, 95% CI: 4·24–122·26, adjusted $P < 0{\cdot}0001$; M–H OR, stratified for race and sex: 17·66; 95% CI: 4·19–74·36, adjusted $P < 0{\cdot}0001$). The combination $KIR2DS2^+/KIR2DL2^+$ was associated inversely with systemic sclerosis (crude OR: 0·29, 95% CI: 0·16–0·53, adjusted $P = 0{\cdot}0001$; M–H OR: 0·31, 95% CI: 0·18–0·54, adjusted $P = 0{\cdot}0003$). Restricting the analyses to patients who fulfilled the ACR criteria, the results were practically unchanged.

Examining individually the profiles of *KIR* genes in the entire sample of patients and controls, 33 different combinations of *KIR* genes were observed. Only one of these profiles (which contained the combination $2DS2^+/2DL2^+$) was more frequent in controls than in patients (OR: 0·11, 95% CI: 0·012–0·48, $P < 0{\cdot}001$). Other profiles were not associated with SSc.

Analysing specifically the *KIR2DS2* gene, it was not related significantly to risk for SSc (Table 2). However, after performing stratified analysis according to the *KIR2DL2* status, *KIR2DS2* was a significant risk factor for systemic sclerosis, particularly in the absence of *KIR2DL2* (Table 4). Furthermore, we observed linkage disequilibrium between absence of *KIR 2DL2* and the presence of *2DS2* ($P < 0{\cdot}0001$), meaning that this combination occurs more frequently in disease than would be expected from a random formation of haplotypes.

The associations of activating and inhibitory *KIR* genes with SSc were analysed additionally in the context of their respective HLA-C ligands using stratified analysis. The odds ratios of *KIR2DL2*, *KIR2DS2*, $KIR2DS2^+/KIR2DL2^-$, $KIR2DS2^-/KIR2DL2^+$ and $KIR2DS2^+/KIR2DL2^+$ for SSc were virtually unchanged after stratification for HLA-C1 status, and no significant interactions were observed. For example, in HLA-C1-negative individuals the odds ratio of *KIR2DL2* for SSc was 0·20 (95% CI: 0·05–0·71), while in HLA-C1-positive individuals it was 0·23 (0·11–0·46). In the same way, the tests for associations of *KIR2DS1*, *KIR2DL1* and its combinations with SSc were changed minimally and non-significantly after stratification for HLA-C2, and there were no significant interactions.

When clinical and laboratory data of the SSc patients were compared, no significant differences in the *KIR* gene frequencies were found with regard to the severity of skin disease, disease subtype, pulmonary interstitial and vascular involvement and autoantibody profile.

Discussion

The results of the present study, investigating a sample of patients and controls from south Brazil, suggests that the *KIR* allele $2DL2^+$ is protective for SSc, while the combination $KIR\ 2DS2^+/2DL2^-$ is related to increased risk for the disease.

Two previous studies have investigated the frequencies of *KIR* genes in SSc patients, reporting discrepant results. Momot *et al.* [10], studying 102 cases and 100 controls, found an association of the combination $KIR\ 2DS2^+/2DL2^-$ with increased risk for SSc in a sample of German SSc patients. This result is confirmed by our study. However, they have not found a significant independent protective role for the *KIR2DL2*. Pellet *et al.* [11], studying 90 Canadian patients and 416 controls, found a marginally significantly (and not adjusted for multiple comparisons) higher prevalence of *KIR2DS1* in SSc cases. Contrasting with this result,

Table 4. Association of *KIR2DS2* and risk of systemic sclerosis stratified by *KIR2DL2* status.

	Systemic sclerosis CF (%)	Healthy controls CF (%)	OR (95% CI)	*P*-value*
$KIR2DL2^+$				
$KIR2DS2^+$	31	66	4·23 (0·50–94·57)	1·0
$KIR2DS2^-$	1	9		
$KIR2DL2^-$				
$KIR2DS2^+$	28	2	10·64 (2·22–69·89)	0·008
$KIR2DS2^-$	50	38		

*Yates' corrected χ^2 or Fisher's exact test, adjusted for multiple comparisons (15 tests; see Table 2). Additional results of the table: Mantel–Haenszel odds ratio: 7·94 [95% confidence interval (CI): 2·35–26·78]; adjusted $P = 0{\cdot}003$. *KIR*, killer immunoglobulin-like receptor.

we found a statistical trend for a lower prevalence *KIR2DS1* in patients. Pellet *et al.* [11] also reported that the presence of at least one of the two activating *KIR* (*KIR2DS1* and/or *2DS2*) was increased significantly in patients (80%) when compared with controls (62%). We were also unable to reproduce this finding, observing 60·0% of *KIR2DS1* and/or *2DS2* in cases and 69·6% in controls.

The main finding from our study was that the inhibitory *KIR2DL2* is a strong protective factor for SSc (OR = 0·22). Furthermore, we observed that the presence of the activating *KIR2DS2* (the corresponding activating counterpart of *KIR2DL2*) is a significant risk for the disease, but only in the absence of *KIR2DL2* (Tables 3 and 4). When *KIR2DS2* was present concomitantly with *KIR2DL2*, protection from disease was observed (Table 3), suggesting that *KIR2DL2* has a dominant protective effect over *KIR2DS2*. This can probably be explained by the interaction between *KIR* and HLA molecules. The most important ligands for inhibitory *KIR* are HLA-C molecules [5]. The HLA binding domains of the corresponding activating KIR are almost identical to the inhibitory KIR binding domains, but have a lower affinity for HLA-Cw [24]. This may be a possible explanation for the preponderance of *KIR2DL2* over *KIR2DS2* that was observed in our data and also shown by Momot *et al.* [10].

Considering the results of Momot *et al.* [10] and ours, it is possible that KIR2DS2 and KIR2DL2 (activating and inhibitory KIRs, respectively) are antagonistic molecules involved in regulation of the activity of NK cells and T cell activation in systemic sclerosis [6]. This combination of genes has also been implicated in the pathogenesis of other rheumatic diseases. In rheumatoid arthritis, the presence of *KIR2DS2* was related to vasculitis [25]. Another study observed an association of *KIR2DS2* in the absence of ligands of *KIR2DL2* with increased risk of psoriatic arthritis [26]. Recent evidence suggests involvement of the combination *KIR2DS2*$^{+}$/*KIR2DL2*$^{-}$ in the pathogenesis of Sjögren's syndrome [27].

In our study, patients and controls presented a statistically significant difference in mean age. However, SSc is relatively rare. The prevalence of SSc is reported to be between 242–286 and 86–233 per million in North America and Australia, respectively, while the incidence is estimated to be around 20 per million per year [28]. Therefore, it is extremely unlikely that a significant number of control individuals will develop SSc in the future.

Considering the high complexity of this gene system, with a great variety of possible genotype profiles, we believe that these observations are physiologically relevant. Despite the differences observed in studies from distinct ethnic groups, they all point to susceptibility and protective roles of certain activating and inhibitory *KIR* genes in SSc. It has become apparent that the effect or function of NK cells is regulated by a balance between opposing signals delivered by the MHC class I-specific inhibitory receptors and by the activating receptors responsible for NK cell triggering.

Following the identification of possible individual genetic determinants of SSc susceptibility, it is necessary to increase the understanding of how these genetic polymorphisms relate to the development of SSc. Biological confirmation of these genetic alterations into functional studies is essential to determine whether these associations are, in fact, causal. Functional studies on the activation of NK cells support the notion of a predominance of inhibitory effects during simultaneous ligation of activating receptors and inhibitory receptors with target cell ligands, resulting usually in down-regulation of the signals that trigger the activating pathways [29]. These observations support further the notion of a possible dominant protective role of some inhibitory *KIR* genes, as we have observed in this study.

In conclusion, our data, combined with previous evidences, point to a significant role of the *KIR* gene system in susceptibility for SSc. Functional studies attempting to dissect the mechanisms involved in the interaction of activating and inhibitory KIR molecules during activation of T and NK cells may yield important insights into the pathogenesis of SSc and other autoimmune diseases.

Disclosure

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