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Combined effects of CXCL8 and CXCR2 gene polymorphisms on susceptibility to systemic sclerosis

Patricia Hartstein Salim [a,*], Mariana Jobim [b], Markus Bredemeier [c], José Arthur Bogo Chies [d], João Carlos Tavares Brenol [c], Luiz Fernando Jobim [e], Ricardo Machado Xavier [c]

[a] Graduate Program in Medical Sciences, Universidade Federal do Rio Grande do Sul. Porto Alegre, RS, Brazil
[b] Department of Immunology, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil
[c] Department of Rheumatology, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil
[d] Department of Genetics, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[e] Department of Internal Medicine, Universidade Federal do Rio Grande do Sul School of Medicine, Porto Alegre, RS, Brazil

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ABSTRACT

A previous study suggested that the CXCR2 (+1208) TT genotype was associated with increased risk of systemic sclerosis (SSc). In the present study, we investigated the influence of variation in the CXCL8 and CXCR2 genes on susceptibility to SSc and combined the variant alleles of these genes to analyze their effects on SSc.

Methods: One fifty one patients with SSc and 147 healthy bone marrow donors were enrolled in a case-control study. Blood was collected for DNA extraction; typing of CXCL8 (−251) T/A and CXCR2 (+1208) T/C genes was made by polymerase chain reaction with sequence specific primers (PCR-SSP), followed by agarose gel electrophoresis.

Results: The CXCR2-TC genotype was significantly less frequent in patients (23.8% versus 55.1% in controls; $P < 0.001$, $OR = 0.26$, 95%CI = 0.15–0.43), whereas the CXCR2-CC genotype was significantly more frequent (44.4% versus 22.4% in controls; $P < 0.001$, $OR = 2.76$, 95%CI, 1.62–4.72). When CXCR2 and CXCL8 combinations were analyzed, the presence of CXCR2 T in the absence of CXCL8 A (CXCR2 T+/CXCL8 A−) was more frequent in patients than in controls (34.5% versus 3.5%; $P < 0.001$, $OR = 14.50$, 95%CI = 5.04–41.40). However, CXCR2 TT and CXCL8 A were significantly more common in controls (100%) than in patients (58.3%) ($P < 0.001$). Likewise, the presence of CXCR2 TC and CXCL8 A was more frequent in controls (95.1%) than in patients (75%) ($P = 0.004$). Furthermore, the CXCR2-CC genotype in CXCL8 A was more frequent in patients (59.7% versus 0% in controls; $P < 0.001$, adjusted $OR = 98.67$, 95%CI = 6.04–1610.8). In patients, a high frequency was observed in combination with the CXCL8 TA and AA genotypes ($P < 0.001$; $OR = 28.92$), whereas in controls, there was a high frequency of combination with CXCL8 T ($P < 0.001$; $OR = 0.03$) and TT ($P < 0.001$; $OR = 0.01$). Conclusions: These findings suggest a protective role of CXCL8 (−251) A in the CXCR2 (+1208) TT and TC genotypes and an increased risk of CXCL8 (−251) A in association with the CXCR2 (+1208) CC genotype in SSc patients.

1. Introduction

Systemic sclerosis (SSc) is a complex disease that occurs in genetically predisposed individuals who have encountered specific environmental exposures and/or other stochastic factors [1]. It is currently believed that the pathogenesis of SSc involves progression from an early inflammatory phase to fibrosis of the connective tissues, affecting the skin and internal organs [2], compounded by immune disturbances suggestive of autoimmunity, which are characterized by the presence of high serum levels of autoantibodies [3].

Emerging data suggest that chemokines may be essential contributors to tissue damage in scleroderma [4]. Chemokine receptors belong to the rhodopsin-like superfamily of G protein-coupled receptor, and their structure consists of seven α-helical transmembrane domains. The term "CXC" comes from the chemokine protein structure, where two N-terminal cysteine residues that form a disulfide bond are separated by another amino acid [5].

IL-8, a proinflammatory cytokine also known as CXCL8, is part of the CXC subgroup. CXCL8 binds to CXCR1 and CXCR2 receptors and has several functions, mainly initiation of chemotaxis; induction of angiogenesis; stimulation of cell proliferation; mediation

* Corresponding author. Address: Hospital de Clínicas de Porto Alegre, Serviço de Reumatologia, 6 andar, sala 630, Rua Ramiro Barcelos 2350, Bairro Bom Fim, CEP 90035-903, Porto Alegre-RS, Brazil. Tel. /fax: +55 51 2101 8340.
E-mail addresses: patysalim@gmail.com (P.H. Salim), rmaxavier@hcpa.ufrgs.br (R.M. Xavier).

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of pain and hypernociception; increase of intracellular calcium concentration in neutrophils, monocytes, T lymphocytes, basophils, eosinophils, and keratinocytes; induction of histamine and leukotriene release; stimulation of prostaglandin E2 production by smooth muscle cells; and induction of leukocyte migration [6].

CXCR2 receptors are expressed on neutrophils, have 77% amino acid identity and bind CXCL8 with high affinity [7]. The CXCR2 gene is located on 2q35, and some of its polymorphisms have been widely investigated in disease association studies. The three most commonly investigated are the +785 polymorphism (C/T) located in exon 3, which does not alter the amino acid sequence of the coded protein, and polymorphisms +1208 (T/C) and +1440 (G/A), which are located at the 3′ untranslated region of the same exon. Polymorphisms in the 3′ untranslated region have the ability to alter the processing, stability and translation of mRNA [8].

A previous study by Renzoni et al. suggested that the presence of CXCR2 (+1208) TT is associated with SSc, and a subgroup analysis revealed that this association is significant regardless of the presence of fibrosing alveolitis. To confirm these findings, the present study was designed to investigate the potential association of CXCR2 and CXCL8 genes with systemic sclerosis [9].

2. Methods

2.1. Patients and controls

151 patients with systemic sclerosis were prospectively evaluated. All patients met the American College of Rheumatology (ACR) criteria for SSc [10] or the criteria suggested by LeRoy and Medsger for diagnosis of early forms of SSc [11]. All patients were Brazilian; most had European and/or African ancestry and lived in the metropolitan area of Porto Alegre, state of Rio Grande do Sul. None of the patients had Asian or Native Brazilian ancestry. Patients with overlapping syndromes were excluded. However, those with a definitive diagnosis of SSc according to the ACR criteria who developed inflammatory myopathy or secondary Sjögren's syndrome were not excluded from the sample. All patients provided written informed consent prior to study enrollment.

The control group comprised 147 healthy voluntary bone marrow donors recruited from the Hospital de Clínicas de Porto Alegre blood bank. All donors were unrelated subjects of European and/or African ancestry, and most lived in the urban area of Porto Alegre. Individuals with any chronic or acute diseases were excluded from the sample, as were those with a family history of genetic diseases (X-linked, autosomal or chromosomal abnormalities). Native Brazilians and subjects with Asian ancestry were not included. All controls provided written informed consent for participation in the study.

3. Clinical evaluation

All patients were interviewed and examined according to an extensive questionnaire designed to assess end-organ damage [12]. Disease subtype was classified as follows: diffuse cutaneous SSc (truncal and acral skin tautness), limited cutaneous SSc (skin tautness restricted to extremities and/or the face), and limited SSc (systemic sclerosis sine scleroderma) [11,13]. Clinical characteristics of the disease were observed and recorded as described elsewhere [12]. Blood samples were collected for serology (antinuclear, anticentromere and antitopoisomerase I antibodies) and DNA extraction. High-resolution computed tomography (HRCT) of the lung was performed in most patients. Doppler echocardiography was used to estimate pulmonary systolic arterial pressure (PSAP), and patients with PSAP $\geqslant$ 40 mm Hg were diagnosed with pulmonary hypertension. The study was approved by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre.

4. Genomic DNA extraction

Genomic DNA was extracted from peripheral blood leukocytes using a modified salting out technique, as described by Miller et al. [14].

4.1. CXCL8. (−251) T/A and CXCR2 (+1208) C/T genotyping

CXCL8 and CXCR2 genes were typed in patients and controls using the polymerase chain reaction with sequence-specific primers (PCR-SSP) method, as described by Sarvestani et al. [15]. As an internal control, β-globin-specific primers were included in the PCR-SSP. For CXCL8 genotyping, 10 μl of PCR reaction mixture—consisting of 250 ng of genomic DNA, 200 mmol/L dNTPs, 2.25 mM MgCl2, 1 × Taq DNA polymerase buffer, 2 units of Taq DNA polymerase, 10 pmol of each test primer, and 5 pmol of internal control primers—were employed. This was followed by a touchdown procedure, which consisted of 25 s at 95 °C, annealing for 45 s at temperatures decreasing from 68 °C (4 cycles) to 61 °C (20 cycles), and an extension step at 72 °C for 40 s. The annealing temperature for the remaining 5 cycles was 58 °C for 40 s. Determination of CXCR2 gene polymorphisms was carried out using the same PCR reaction mixture, except that the concentration of MgCl2 was 1.7 mM. The touchdown procedure was also similar to CXCL8 genotyping, but using annealing temperatures of 70, 65, and 55 °C in the three consecutive steps respectively. The reaction products of CXCL8 and CXCR2 gene amplification were separated on 2.5% agarose gel and stained with ethidium bromide.

4.2. Statistical analysis

Data were analyzed using the EPI-INFO 6.0 and SPSS 11.0 software packages. Carrier frequencies (CF) for the alleles and their combinations were calculated and expressed as percentages. CFs were compared using Yates's chi-squared test or Fisher's exact test. Student's *t* test and the Mann–Whitney *U* were used for between-group comparisons in which the dependent variables were parametric and non-parametric, respectively. Holm's procedure for adjustment of P-values for multiple comparisons was applied with the aid of the WinPepi software. Crude and Mantel–Haenszel odds ratios (OR), with 95% confidence intervals, were calculated for alleles or combinations whose CF distributions were significantly different between patients and controls. The sample size was calculated, using the WinPepi software, for a significance level of 0.05 and a statistical power of 93.71%.

5. Results

The genotype distribution and allele frequencies for the CXCL8 (−251) T/A and CXCR2 (+1208) C/T polymorphisms in SSc patients and controls are presented in Table 1. The allele frequencies of CXCL8 and CXCR2 genes were in Hardy–Weinberg equilibrium in patients and controls alike. No significant difference was found in the frequencies of the CXCL8 (−251) T/A and CXCR2 (+1208) C/T alleles.

In our study, we observed a high frequency of CXCR2 (+1208) CC carrier status in SSc patients (44.4% vs 22.4% in controls; $OR = 2.76$; 95%CI = 1.62–4.72; $P < 0.001$) and a low frequency of CXCR2 (+1208) TC carrier status (55.1% in controls vs. 23.8% in SSc patients; $OR = 0.26$; 95%CI = 0.15–0.43; $P < 0.001$). There were no significant differences between SSc patients and controls with regard to frequency of the CXCL8 (−251) TT, TA and AA genotypes. However, we did observe a high frequency of the CXCL8 (−251) *T* allele in association with the CXCR2 (+1208) CC genotype in controls (100% in controls and 70.1% in SSc; adjusted $OR = 0.03$;

Table 1
Alleles and genotype distribution of the CXCR2 and CXCL8 single-nucleotide polymorphisms in the systemic sclerosis (151) and healthy controls (147)

	Healthy control		Systematic sclerosis		*P*-value*
	n	%	*n*	%	
CXCR21208)					
T	147	50.0	132	43.7	NS
C	147	50.0	170	56.3	NS
TT	33	22.4	48	31.8	
TC	81	55.1	36	23.8	<0.001
CC	33	22.4	67	44.4	
CXCL8251)					
T	146	49.7	167	55.3	NS
A	148	50.3	135	44.7	NS
TT	37	25.2	56	37.1	
TA	72	49.0	55	36.4	NS
AA	38	25.9	40	26.5	

* *P*-values of alleles were calculated by Fischer's exact test and genotypes by chi-squared test.

Table 2
Correlation between CXCR2 and CXCL8 in healthy controls (147) and systematic sclerosis (151).

	Healthy control		Systemic sclerosis		*P*-value*
	n	%	*n*	%	
CXCR2 TT					
CXCL8 T+	18	54.5	33	68.8	NS
CXCL8 A+	33	100.0	28	58.3	<0.001
CXCL8 TT	0	0.0	20	41.7	<0.001
CXCL8 TA	18	54.5	13	27.1	
CXCL8 AA	15	45.5	15	31.3	
CXCR2 TC					
CXCL8 T+	58	71.6	31	86.1	NS
CXCL8 A+	77	95.1	27	75.0	0.004
CXCL8 TT	4	4.9	9	25.0	0.003
CXCL8 TA	54	66.7	22	61.1	
CXCL8 AA	23	28.4	5	13.9	
CXCR2 CC					
CXCL8 T+	33	100.0	47	70.1	<0.001
CXCL8 A+	0	0.0	40	59.7	<0.001
CXCL8 TT	33	100.0	27	40.3	<0.001
CXCL8 TA	0	0.0	20	29.9	
CXCL8 AA	0	0.0	20	29.9	

* Chi-Square Test or Fischer's Exact Test.

95%CI = 0.0–0.57; $P < 0.001$), in Table 2. Likewise, the combination of the CXCL8 (−251) TT and CXCR2 (+1208) CC genotypes was more frequent in healthy controls than in SSc patients (100% vs 40.3% respectively; adjusted $OR = 0.01$; 95%CI = 0.0–0.17; $P < 0.001$).

Furthermore, when we investigated the presence of the CXCL8 (−251) *A* allele in the CXCR2 (+1208) CC genotype, we found a high frequency in patients as compared to controls (59.7% vs. 0% respectively; adjusted $OR = 98.67$; 95%CI = 6.04–1610.8; $P < 0.001$). The same was observed in the combination of the CXCL8 (−251) TA and AA with the CXCR2 (+1208) CC genotype (29.9% in SSc vs. 0% in controls; adjusted $OR = 28.92$; 95%CI = 1.76–474.8; $P < 0.001$).

Association of the CXCR2 (+1208) TT genotype with the *A* allele of the CXCL8 (−251) gene was less frequent in patients than in controls (58.3% vs. 100% respectively; adjusted $OR = 0.04$, 95%CI = 0.0–0.32, $P < 0.001$). Similarly, the combination of the CXCL8 (−251) TA genotype and CXCR2 (+1208) TT genotype was less frequent in patients (27.1% vs. 54.5% in controls; $OR = 0.31$; 95%CI = 0.11–0.87; $P = 0.02$). Conversely, the combination of the CXCL8 (−251) TT genotype and CXCR2 (+1208) TT genotype was more frequent in SSc patients (41.7% vs. 0% in controls; adjusted $OR = 48.19$; 95%CI = 2.91–798.7; $P < 0.001$). Finally, presence of CXCR2 + 1208 T in the absence of CXCL8–251 A (CXCR2 T+/CXCL8 A-) was more frequent in patients than in controls (34.5% versus 3.5% respectively; $P < 0.001$, $OR = 14.50$, 95%CI = 5.04–41.40). No significant between-group differences were found in the combination of the CXCL8 (−251) AA genotype and CXCL8 (−251) *T* allele with the CXCR2 (+1208) TT genotype.

Presence of the CXCL8 (−251) *A* allele in the CXCR2 (+1208) TC genotype was significantly less frequent in patients as compared to controls (75.0% and 95.1% respectively; $OR = 0.16$, 95%CI = 0.03–0.63, $P = 0.004$). Furthermore, the combination of the CXCL8 (-251) TT genotype and CXCR2 (+1208) TC genotype was more frequent in SSc patients than in controls (25% vs. 4.9% respectively; $OR = 6.42$, 95%CI = 1.6–30.3, $P = 0.003$). No association was observed with the CXCL8 (−251) T / TT / AA genotype in combination with the CXCR2 (+1208) TC genotype.

When clinical and laboratory data of the SSc patients were compared, no significant differences in CXCL8 (−251) and CXCR2 (+1208) gene frequencies were found with regard to the severity of skin disease, disease subtype, interstitial and vascular pulmonary involvement, or autoantibody profile.

6. Discussion

The approximately 50 known human chemokines are grouped into four families on the basis of conserved cysteine residues near their N-terminus, and are designated as the CC, CXC, C, and CX3C subfamilies. The CXC chemokine ligands are further subdivided on the basis of the presence or absence of another three-amino acid sequence, glutamic acid–leucine–arginine (the ELR sequence motif), immediately proximal to the CXC sequence [16]. The ELR-positive CXC chemokines, which include interleukin-8 (CXCL8), are potent neutrophil chemoattractants. The chemokine-receptor binding initiates a complex signaling cascade that generates chemotactic responses, degranulation, release of ROS and changes in the affinity of cell surface integrins. [17]. CXCR1 and CXCR2 are both expressed by endothelial cells, but only the expression of CXCR2 is required for endothelial cell chemotaxis [18]. When the function of CXCR2 is blocked, the response of endothelial cells to CXCL8 is abrogated [19].

The importance of CXCL8 receptors for neutrophil migration was demonstrated in a study with IL-8 receptor homologue knockout mice. In these animals, neutrophils failed to cross the epithelium and accumulate in the subepithelial tissue [20]. In addition, overexpression of CXCR2 has been considered an important factor in inflammatory diseases such as rheumatoid arthritis, atherosclerosis and psoriasis [21].

The CXCR2 gene polymorphism +1208 (T/C) has been investigated as a factor in susceptibility to development of multiple sclerosis in Iranian individuals, but no significant association was found [22]. The polymorphisms +785 (C/T) and +1208 (T/C) also showed no positive association with Behçet's disease [23,24]. A similar result was obtained by Breunis et al. [25], who found no relationship between polymorphisms +1208 (T/C) or +1440 (G/A) and Kawasaki disease. It should be noted that children with Kawasaki disease exhibit very high levels of proinflammatory cytokines and chemokines, such as CXCL8 [26].

However, CXCR2 gene polymorphisms appear to be important to the pathogenesis of diseases such as systemic sclerosis-associated fibrosing alveolitis, because there was a significantly higher frequency of individuals with genotypes +785 CC and +1208 TT in a group with this disease [9]. When frequency of the +785

(C/T) polymorphism was evaluated in respiratory diseases, the presence of the *T* allele was considered as a possible protective factor against pulmonary inflammation [27]. A similar protective effect was observed in relation to polymorphisms +1208 (T/C) and +1440 (G/A), and the presence of diplotype 1208T/1440G represented a decreased risk of development of classic Kaposi's sarcoma (*OR* = 0.49, 95%CI = 0.30–0.78) [28].

Several polymorphisms in a given individual may contribute to the individual risk of developing the disease. In our study, presence the of CXCR2 *T* allele in the absence of the CXCL8 *A* allele (CXCR2 T+/CXCL8 A−) was more frequent in patients than in controls (*OR* = 14.50), suggesting an increased risk for the disease. The inverse was also true: the CXCL8 (−251) *A* allele and TA genotype in combination with CXCR2 (+1208) TT genotype were significantly more frequent in healthy controls (*OR* = 0.04 and *OR* = 0.31, respectively), suggesting a protective effect. Conversely, the CXCL8 (−251) TT genotype in association with CXCR2 (+1208) TT genotype was more frequent in SSc patients and suggested higher risk of the disease (*OR* = 48.19).

The CXCR2 (+1208) T/C has the potential of altering mRNA processing, stability or translation and is located in the 3′ untranslated region of exon 3 [8]. A previous study by Renzoni et al. [9] reported an association between the presence of CXCR2 + 1208 *T* allele and SSc (*OR* = 1.63). They also found this association to be greater when the allele is homozygous (*OR* = 2.67). These findings were not confirmed in our study. We found a significantly higher risk for systemic sclerosis among carriers of the CXCR2 (+1208) CC genotype (*OR* = 2.76) and a protective effect for carriers of the CXCR2 (+1208) TC genotype (*OR* = 0.26). Furthermore, we observed a significantly higher risk in the CXCR2 (+1208) CC genotype with CXCL8 (−251) *A* allele (*OR* = 98.67), as well as in combination with the CXCL8 (−251) TA genotype (*OR* = 28.92) and CXCL8 (−251) AA genotype (*OR* = 28.92). Conversely, the CXCL8 (−251) *T* allele and TT genotype in combination with CXCR2 (+1208) CC was more frequent in healthy controls, suggesting a protective effect (*OR* = 0.03 and *OR* = 0.01, respectively). Moreover, association between the CXCR2 (+1208) TC genotype and CXCL8 (−251) *A* allele was more frequent in healthy controls (*OR* = 0.16), and association with the CXCL8 (−251) TT genotype was more frequent in SSc patients (*OR* = 6.42). Further studies are still required to improve the understanding of how these genetic polymorphisms relate to the development of SSc.

Renzoni et al. [9] strongly suggested a role for CXCR2 polymorphisms in the pathogenesis of SSc. Their study described the association between CXCR2 and SSc in the British population, indicating that TT homozygosity is a risk factor for the disease. Our study did not corroborate this finding; we identified CC homozygosity as a potential risk factor. The Brazilian population is known to be highly diverse. Intermarriage between the Portuguese and Native Brazilians or African slaves was common in the first centuries of colonization. While the Portuguese people were historically the major European ethnic group in Brazil, subsequent waves of immigration have also contributed to the establishment of a multiethnic population [29]. This would be a plausible explanation for the difference between our findings and those of Renzoni et al. [9]. At any rate, our study evaluated the interaction between CXCL8 and its receptor, CXCR2, while Renzoni et al. [9] did not.

To date, no study has described an association between presence of the CXCL8 (−251) promoter gene and SSc, and only Renzoni et al. [9] have investigated the frequency of CXCR2 (+1208) in patients with SSc. In conclusion, we suggest that CXCR2 (+1208) in association with CXCL8 (−251) may play a role in the pathogenesis of this disease. The presence of the *A* allele (CXCL8) in association with the *T* allele (CXCR2) may contribute to protection against SSc, while its absence may increase the risk of this disease. The C allele of CXCR2 is inversely associated: the presence of the *A* allele of CXCL8 increases the risk of SSc, whereas its absence protects against the disease. Additional studies of other ethnically diverse populations should be performed to ascertain whether this gene could be a marker of SSc. Furthermore, other studies investigating the functionality of the CXCR2 and CXCL8 polymorphisms will be required to achieve a better understanding of the results of disease association studies.

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