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Evidence of Potential Interaction of Chemokine Genes in Susceptibility to Systemic Sclerosis

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Objective. **To examine genetic polymorphisms in the chemokine pathway, and to assess their interactions in relation to susceptibility to systemic sclerosis (SSc).**

Methods. **To identify the risk of SSc conferred by genetic polymorphisms in the chemokine pathway, 10 single-nucleotide polymorphisms (SNPs) from 8 candidate genes were studied in 99 patients with SSc and 198 age- and sex-matched controls in a Korean population. SNPs were genotyped by polymerase chain reaction–restriction fragment length polymorphism or sequence-specific primer methods. Genetic associations between each SNP and SSc risk, calculated as odds ratios with 95% confidence intervals, were estimated using chi-square tests. Haplotypes for the 2 polymorphisms in the gene CCL5 (RANTES) were constructed, and their associations with SSc were tested. Gene–gene interactions were investigated using a recently described novel method, and the results were confirmed by conditional logistic regression. Adjustment for multiple testing was based on Bonferroni correction.**

Results. **There was significant evidence of gene–gene interaction between polymorphisms in the genes CXCL8 (interleukin-8) and CCL5, and both of these were associated with an increased risk of SSc. This SNP–SNP interaction was confirmed by 2 independent statistical methods. The associations remained significant after Bonferroni adjustment for multiple testing. No significant association between each individual SNP or haplotype and the risk of SSc was found.**

Conclusion. **Crosstalk between the 2 chemokines CXCL8 and CCL5 may contribute to the susceptibility to SSc.**

Systemic sclerosis (SSc; scleroderma) is an autoimmune inflammatory disorder characterized by progressive cutaneous and visceral fibrosis, vascular abnormalities, and immune system abnormalities (1). Several lines of evidence suggest a complex genetic etiology for the susceptibility to SSc (2,3). For instance, a positive family history of SSc confers the strongest relative risk for this disease (4). The concordance rate for antinuclear antibodies was found to be significantly higher in monozygotic twins compared with dizygotic twins (2,5). However, the genetic basis of SSc remains elusive. Elucidation of the genetic mechanisms underlying SSc will help to unravel the pathophysiologic mechanisms of this complex disorder, and thus will contribute to the design and development of effective targeted therapies.

Chemokines are a large family of small (7–15-kd), structurally related heparin-binding proteins that may mediate leukocyte–endothelium interactions and cell transmigration, leading to triggering of abnormal alterations in scleroderma (6). The initiation and progression of SSc involves multiple chemokines and inflammatory cells such as T cells, macrophages, dendritic cells, eosinophils, and mast cells (6). The complex interactions between chemokines and inflammatory cells stimulate the overproduction of extracellular matrix protein synthesis by fibroblasts (7,8). Therefore, chemokines are of fundamental importance in the pathogenesis of SSc (9).

Genetic variations in a variety of chemokine genes, such as CCL2 (monocyte chemoattractant protein 1 [MCP-1]) (10) and CXCR2, and their associations with SSc have been reported (2). However, these reported

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Table 1. Primers and restriction enzymes used for genotyping of chemokine genes

Chemokine	Location	Primers, forward and reverse
CXCL8	−353 A/T	5′-GAATTCAGTAACCCAGGCAT-3′ and 5′-AAGCTTGTGTGCTCTGCTGTCTCT-3′
CXCR1	+827 G/C	5′-CCAGGTGATCCAGGAGAG/C-3′ and 5′-TCAGAGGGTTGGAAGAGACATT-3′
CXCR2	+786 C/T	5′-CGTCCTCATCTTCCTGCTC/T-3′ and 5′-GGAGTCCATGGCGAAGCTTC-3′
CCL2	−2518 G/A	5′-CCGAGATGTTCCCAGCACAG-3′ and 5′-CTGCTTTGCTTGTGCCTCTT-3′
CCR2	+64 V/I	5′-TTGTGGGCAACATGATGG-3′ and 5′-CTGTGAATAATTTGCACATTGC-3′
CCL5	−403 G/A	5′-GCCTCAATTTACAGTGTG-3′ and 5′-TGCTTATTCATTACAGATGTT-3′
	−28 G/A	5′-ACAGAGACTCGAATTTCCGGA-3′ and 5′-CCACGTGCTGTCTTGATCCTC-3′
CCL3	+113 C/T	5′-CACGTGAGTCTGAGTTTC-3′ and 5′-GTTCTCTTATCTCAGTTC-3′
CCL3	+459 C/T	5′-CACGTGAGTCTGAGTTTC-3′ and 5′-GTCTGGTTCAAGAAGTCATACCCCAACCCAAGAGAG-3′
CCR5	Δ32	5′-TTTACCAGATCTCAAAAAGAAG-3′ and 5′-GGAGAAGGACAATGTTGTAGG-3′

associations rarely have been replicated in independent cohorts. In the present study, we investigated the association between genetic polymorphisms in 8 chemokines or chemokine receptors and the risk of SSc, and assessed their interactions in 99 patients with SSc and 198 age- and sex-matched healthy controls in a homogeneous Korean population.

PATIENTS AND METHODS

Study population. A total of 99 patients with SSc were enrolled at the Rheumatology Clinic of Seoul National University Hospital between March 1999 and December 2002. The diagnosis of SSc was made according to the American College of Rheumatology (formerly, the American Rheumatism Association) classification criteria (11). We obtained the following information on each patient: demographic data, duration of the disease, cutaneous disease type, and autoantibody status. We also enrolled 198 age- and sex-matched healthy control subjects. Demographic data on the healthy controls were also obtained. Blood samples were obtained from each patient and healthy control, after informed consent was obtained from all subjects. The study was approved by the institutional review board of Seoul National University Hospital.

Genotyping. Genomic DNA was extracted from the peripheral blood of all subjects, using the QIAamp Blood kit (Qiagen, Valencia, CA). Genotyping was carried out in accordance with published methods (12–17). A polymerase chain reaction (PCR)–sequence-specific primer method was used to genotype CXCR1 +827 G/C and CXCR2 +786 C/T, while a PCR–restriction fragment length polymorphism method was used to genotype CXCL8 (interleukin-8 [IL-8]) −353 A/T, CCL2 (MCP-1) −2518 G/A, CCR2 +64 V/I, CCL5 (RANTES) −403 G/A, CCL5 −28 G/A, CCL3 (macrophage inflammatory protein 1α) +113C/T, and CCL3 +459 C/T polymorphisms. The length of the PCR product was compared with the genotype CCR5 Δ32 polymorphism. Table 1 lists all of the PCR primers used in this study.

Genetic association analyses. We used chi-square tests with 1 degree of freedom to test Hardy-Weinberg equilibrium (HWE) for each SNP among the controls (18). Allele and genotype frequencies for each individual polymorphism were calculated by gene counting. The associations between each SNP and SSc risk were determined by chi-square test. When performing association analyses for each polymorphism, we assumed 3 models: recessive, additive, and dominant effects models. In the recessive model, we tested the effect of the minor allele homozygotes against all other individual allele combinations. In the additive model, we tested the effect of allele heterozygotes versus major-allele homozygotes (1 copy) or minor-allele homozygotes versus major-allele homozygotes (2 copies). In the dominant model, both heterozygote variants and rare homozygote variants were combined into a single group for comparison with other groups. Odds ratios (ORs) and the corresponding 95% confidence intervals (95% CIs) were estimated for each SNP under each of these 3 models of association.

Since haplotype analysis may provide more power to detect an association than would single-marker analysis alone (19), we also constructed haplotypes for the 2 polymorphisms in the CCL5 (RANTES) gene in all patients and controls, using the program PHASE 2.0 (20,21). Haplotypes with frequencies less than or equal to 0.01 were combined with the most similar haplotypes. Analyses of haplotype associations were performed by comparing frequencies of each haplotype against all other haplotypes present within our study sample.

Detection of gene–gene interactions. We tested all SNP–SNP interactions using 2 methods. We first used a novel method to identify all of the pairwise gene–gene interactions involved in SSc, as recently described (22). The principle of this statistical method is based on the difference in levels of linkage disequilibrium (LD) between patients and controls. This LD-based statistical method has been shown to be more powerful than regression-based methods in detecting gene–gene interactions. We then confirmed the interaction using conditional logistic regression analysis. *P* values were adjusted for multiple testing using the most conservative method, the Bonferroni correction.

RESULTS

Characteristics of the study population. Table 2 presents the characteristics of the SSc study population. Approximately 89% of the patients with SSc were female. The mean ± SD age of the patients was 46.0 ± 12.2 years, and the mean ± SD duration of disease was 5.0 ± 4.6 years. Almost one-half of the 99 patients with SSc had the diffuse form of the disease, and 55% of the

Table 2. Characteristics of the 99 patients with systemic sclerosis

Age, years	46.0 ± 12.2
Sex, %	
Male	11.1
Female	88.9
Duration of disease, years	5.0 ± 4.6
Cutaneous type, %	
Diffuse	49.5
Limited	50.5
Autoantibody positivity, %	
Anti–Scl-70	54.5
Anticentromere	2.0

* Except where indicated otherwise, values are the mean ± SD.

patients had anti–Scl-70 antibodies. Eighty-four percent of the SSc patients had interstitial lung disease.

Genetic associations. All of the SNPs were in HWE. SNPs in the genes CCR5 and CCL3 −113 were not polymorphic in our study population, and therefore these were excluded from further analyses.

Three SNPs showed significant associations with SSc. Under the dominant effects model, CXCR2 (OR 1.7, 95% CI 1.0–2.7, $P = 0.04$) and CCL5 (OR 2.0, 95% CI 1.2–3.3, $P = 0.006$) were found to be significantly associated with SSc. Under the additive model, CCR2 was significantly associated with the risk of SSc (OR 3.2, 95% CI 1.2–8.7, $P = 0.02$). However, after adjustment for multiple comparisons using Bonferroni correction, all of these positive associations with SSc became nonsignificant. Stratification of patients according to their disease severity (i.e., diffuse cutaneous SSc versus limited cutaneous SSc) led to results similar to those obtained in the whole group (results not shown). All other SNPs showed no association with SSc.

Findings of allele-based genetic association analyses under the recessive, additive, and dominant effects models are summarized in Table 3. In the CCL5 (RANTES) gene, three 2-SNP haplotypes were identified, and their frequencies are presented in Table 4. None of these haplotypes was significantly associated with the risk of SSc in our study population.

Evidence of gene–gene interactions. We identified a significant gene–gene interaction between polymorphisms in the genes CXCL8 and CCL5 −403, both by the LD-based statistical method ($P = 2.54 \times 10^{-11}$) and by logistic regression analysis ($P = 0.0014$). This

Table 3. Frequencies of genotypes and alleles and results of tests for associations with disease risk among chemokine genes in 99 patients with systemic sclerosis and 198 age- and sex-matched controls*

	Genotype			Alleles		Model of association†			
Gene, group	1/1	1/2	2/2	Allele 1	Allele 2	Recessive	Additive 1 copy	Additive 2 copies	Dominant
CXCL8									
Controls	79 (0.40)	95 (0.48)	24 (0.12)	253 (0.63)	143 (0.37)	1.2 (0.6–2.4); 0.62	0.9 (0.6–1.6); 0.91	1.2 (0.5–2.5); 0.87	1.0 (0.6–1.7); 0.96
Patients	40 (0.41)	45 (0.45)	14 (0.14)	125 (0.64)	73 (0.36)				
CXCR1									
Controls	159 (0.80)	39 (0.19)	0 (0.00)	357 (0.90)	39 (0.10)	2.0 (0.1–32); 0.80	0.5 (0.3–1.1); 0.09	NA	1.8 (0.9–3.6); 0.14
Patients	87 (0.88)	11 (0.11)	1 (0.01)	185 (0.93)	13 (0.07)				
CXCR2									
Controls	93 (0.47)	77 (0.39)	28 (0.14)	263 (0.66)	133 (0.34)	0.7 (0.3–1.5); 0.42	0.6 (0.4–1.0); 0.09	0.6 (0.3–1.2); 0.21	1.7 (1.0–2.7); 0.04
Patients	59 (0.60)	30 (0.30)	10 (0.10)	148 (0.75)	50 (0.25)				
CCR2									
Controls	108 (0.55)	82 (0.41)	8 (0.04)	298 (0.75)	98 (0.25)	2.7 (1.0–7.0); 0.07	1.5 (0.9–2.4); 0.17	3.2 (1.2–8.7); 0.02	0.6 (0.4–1.0); 0.05
Patients	42 (0.43)	47 (0.47)	10 (0.10)	131 (0.66)	67 (0.34)				
CCL2									
Controls	78 (0.39)	37 (0.51)	13 (0.10)	256 (0.65)	140 (0.35)	1.3 (0.6–2.8); 0.56	0.6 (0.4–1.0); 0.06	1.0 (0.5–2.3); 0.91	1.5 (0.9–2.5); 0.12
Patients	48 (0.49)	37 (0.38)	13 (0.13)	133 (0.68)	63 (0.32)				
CCL3 −459									
Controls	70 (0.35)	91 (0.46)	37 (0.19)	231 (0.58)	165 (0.42)	0.8 (0.4–1.5); 0.55	0.7 (0.4–1.2); 0.23	0.6 (0.3–1.3); 0.30	1.5 (0.9–2.4); 0.13
Patients	44 (0.44)	40 (0.40)	15 (0.15)	128 (0.65)	70 (0.35)				
CCL5 −28									
Controls	129 (0.65)	62 (0.31)	7 (0.04)	320 (0.81)	76 (0.19)	1.1 (0.3–4.0); 0.91	0.7 (0.4–1.3); 0.35	1.0 (0.3–3.7); 0.81	1.3 (0.8–2.2); 0.33
Patients	70 (0.71)	25 (0.25)	4 (0.04)	165 (0.83)	33 (0.17)				
CCL5 −403									
Controls	67 (0.34)	100 (0.51)	31 (0.15)	234 (0.59)	162 (0.41)	0.7 (0.3–1.4); 0.38	0.5 (0.3–0.9); 0.02	0.5 (0.2–1.0); 0.09	2.0 (1.2–3.3); 0.0056
Patients	50 (0.51)	38 (0.38)	11 (0.11)	138 (0.70)	60 (0.30)				

* Except where indicated otherwise, values are the number (frequency) of each genotype or allele variant. NA = not applicable.
† Values are the odds ratios (95% confidence intervals); *P* value.

Table 4. Frequency of CCL5 haplotypes and risk of systemic sclerosis associated with these haplotypes*

Haplotype	Controls	Patients	OR (95% CI)
CA	88 (0.22)	34 (0.17)	0.8 (0.5–1.2)
CG	232 (0.58)	131 (0.66)	1.1 (0.9–1.5)
GA	76 (0.20)	33 (0.17)	0.9 (0.6–1.4)

* Except where indicated otherwise, values are the number (frequency) of each haplotype. OR = odds ratio; 95% CI = 95% confidence interval.

gene–gene interaction remained significant after correction for multiple comparisons ($P < 0.0001$ and $P = 0.039$ by LD-based statistical test and logistic regression analysis, respectively, with Bonferroni correction).

DISCUSSION

This study provides strong evidence to demonstrate, for the first time, a significant gene–gene interaction between polymorphisms in the genes CXCL8 and CCL5 as assessed by 2 independent methods, a novel, LD-based statistical method and conditional logistic regression analysis. This gene–gene interaction remained highly significant even after correction for multiple testing.

In patients with SSc, a multisystem connective tissue disorder characterized by excessive fibrosis, vascular abnormalities, and immune system dysfunction (1,7), the clinical manifestations are highly heterogeneous, ranging from mild limited skin sclerosis with minimal organ involvement to diffuse skin involvement and severe fibrosis of multiple internal organs. The onset and development of SSc requires complicated crosstalk among multiple biologic pathways, involving inflammatory cytokines, chemokines and their receptors, and growth factors (1,6–9). For example, proinflammatory cytokines, such as IL-1, IL-2, interferon-γ, and tumor necrosis factor α (TNFα), can induce the production of chemokines (23). Macrophages may produce a broad variety of chemokines, including IL-8, CCL2, and CCL3 (24). In addition, lymphocytes and fibroblasts can produce various chemokines after targeted stimulation (24,25). Conversely, chemokines may also stimulate the release of proinflammatory cytokines. For instance, CCL3 can stimulate the synthesis of IL-1, IL-6, and TNFα (26), and CCL5 can induce the production of IL-6 and IL-8 (27).

Although the exact mechanisms involved in the pathogenesis of SSc are poorly understood, it is evident that these mechanisms are highly complex. Both genetic and environmental factors, and their complex interactions, are involved in the disease process (1–3,7). Moreover, the clinical manifestations of SSc are a result of a complex interplay among multiple components that are organized hierarchically into multiple biologic pathways.

Chemokines such as CXCL8 and CCL5, and their receptors, are key contributors to tissue damage in SSc, potentially by directing the migration of proinflammatory cells into the affected areas (6). Increased levels of CXCL8 protein have been observed in SSc skin biopsy specimens (28) and in bronchoalveolar lavage fluid from patients with SSc (29). Scleroderma fibroblasts cultured in vitro were found to produce more CXCL8 than that in normal fibroblasts (30). Serum concentrations of CXCL8 were significantly higher in patients with SSc than in healthy controls (31). Strong expression of CCL5 has been reported in the epidermis of scleroderma skin, both at the messenger RNA level and at the protein level, but not in the skin of healthy subjects (32). Blockade of CCL5 using neutralizing antibodies inhibited its migration toward sites of skin lesions (33).

Patients with SSc have a strong genetic predisposition (2,3,7). Previous studies have identified a variety of genetic polymorphisms in the genes encoding chemokines, and have found significant associations of these polymorphisms with the risk of SSc. For example, Karrer et al (10) found that the frequency of the GG homozygote in the CCL2 gene promoter was significantly higher in patients with SSc than in controls. Polymorphisms in the gene CXCR2 were associated with SSc in a Caucasian population (12). SNPs in the gene IL-1α were reported to be strongly related to the development of SSc (34,35). Genetic polymorphisms in other genes, such as transforming growth factor β1 (36,37), TNFα (38,39), endothelial nitric oxide synthase (eNOS) (40,41), angiotensin-converting enzyme (ACE) (40,42), and SPARC (43,44), and their implications in the pathogenesis of SSc have also been investigated.

However, the mechanisms of action leading to SSc susceptibility are very complex. Results of association studies have suggested that SSc susceptibility may be determined by a number of different genes, with various genes interacting to produce the different subtypes of SSc. Furthermore, association studies have often yielded conflicting results. Many of the reported associations have not been replicated in independent studies. For instance, Kawaguchi et al (35) found that the C allele of the -889 SNP in the IL-1α gene increased the risk of SSc in a Japanese population. However, this finding could not be replicated in another study using a Slovak Caucasian population. Instead, the

T allele frequency of the same SNP in IL-1α was found to be significantly higher in SSc patients compared with healthy controls (34). Data on the association of the genes eNOS (41,42), ACE (40,42), and SPARC (43,44) with SSc have also been conflicting. Potential reasons for these discrepant findings from association studies include differences in population stratification, allele and locus heterogeneity, heterogeneous clinical presentation and disease course, a lack of power in the statistical methods used, and/or insufficient sample sizes (2). However, failure to account for gene–gene interactions between or within multiple biologic pathways has been recently proposed as one of the most important causes for the conflicting findings (45,46).

CXCL8 and CCL5 are involved in the same chemokine biologic pathway. They may function synergistically in the initiation and progression of SSc. The observed interaction between polymorphisms in these 2 chemokines could be related to their chemotactic abilities in directing the migration of proinflammatory cells to the affected spaces (47). In this study, we also identified 3 SNPs that are significantly associated with SSc under different genetic models: CXCR2 under a dominant model, CCR2 under an additive model, and CCL5 under a dominant model. However, all of these associations with SSc became nonsignificant after correction for multiple testing, and thus may represent false-positive findings.

The samples in this study comprised 99 patients and 198 controls. Although this number of patients may be considered too small if one is studying a common disease such as asthma or hypertension, different criteria should be applied to establish genetic associations in rare diseases such as SSc. According to a recent report by Kraft et al (48), the power calculation for a rare disease with low population prevalence should be different from that for a common disorder with high population prevalence. Therefore, we speculate that the sample size required for detecting gene–gene interactions in SSc, which has a prevalence of ~2–30 per million in the Asian population, could be much lower than that for common disorders with high population prevalence.

The data presented in the present report are preliminary. Studies of patients from large, diverse populations are needed to confirm the genetic interaction identified in this study. Animal models of SSc (49) may be particularly useful in elucidating the possible pathways involved in the interactions of the chemokines CXCL8 and CCL5.

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AUTHOR CONTRIBUTIONS

Dr. Song had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study design. Lee, Song.
Acquisition of data. Lee, Kim.
Analysis and interpretation of data. Lee, Zhao, Song.
Manuscript preparation. Lee, Zhao.
Statistical analysis. Zhao, Xiong.

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