

Phenotype-Haplotype Correlation of *IRF5* in Systemic Sclerosis: Role of 2 Haplotypes in Disease Severity

PHILIPPE DIEUDE, KAREN DAWIDOWICZ, MICKAËL GUEDJ, YONA LEGRAIN, JULIEN WIPFF, ERIC HACHULLA, ELISABETH DIOT, JEAN SIBILIA, LUC MOUTHON, JEAN CABANE, ZAHIR AMOURA, JEAN-LUC CRAKOWSKI, PATRICK CARPENTIER, JEROME AVOUAC, OLIVIER MEYER, ANDRE KAHAN, CATHERINE BOILEAU, and YANNICK ALLANORE

ABSTRACT. Objective. Identification of an association between *IRF5* rs2004640 and systemic sclerosis (SSc) has highlighted a key role for type 1 interferon (IFN). Additional functional *IRF5* variants have been identified as autoimmune susceptibility factors. Our aim was to investigate whether *IRF5* haplotypes confer susceptibility to SSc, and to perform genotype haplotype-phenotype correlation analyses.

Methods. We genotyped *IRF5* rs377385, rs2004640, and rs10954213 in 1623 individuals of French European Caucasian origin. SSc patient subphenotypes were analyzed according to cutaneous subsets and for SSc-related pulmonary fibrosis.

Results. Case-control studies of single markers revealed an association between *IRF5* rs3757385, rs2004640, and rs10954213 variants and SSc. We identified an *IRF5* risk haplotype “R” ($p_{\text{adj}} = 0.024$, OR 1.23, 95% CI 1.07–1.40) and a mirrored protective haplotype “P” ($p_{\text{adj}} = 8.8 \times 10^{-3}$, OR 0.78, 95% CI 0.68–0.90) for SSc susceptibility. Genotype-phenotype correlation analyses failed to detect any association with a single marker. By contrast, phenotype-haplotype correlation analysis was able to detect intra-cohort association and to discriminate SSc patients with from those without the following clinical traits: “R” and/or “P” haplotypes identified diffuse cutaneous SSc ($p = 0.0081$) and fibrosing alveolitis ($p = 0.018$).

Conclusion. *IRF5* haplotypes are more informative than single markers, suggesting that they could be helpful for risk stratification of SSc patients. Our study provides further evidence of a key role of *IRF5* in SSc severity. (First Release March 15 2010; J Rheumatol 2010;37:987–92; doi:10.3899/jrheum.091163)

Key Indexing Terms:

IRF5 INTERFERON HAPLOTYPE SYSTEMIC SCLEROSIS

From the Service de Rhumatologie, Hôpital Bichat Claude Bernard, APHP, Université Diderot-Paris 7, Paris; Laboratoire Statistique et Génome, UMR CNRS-8071 / INRA-1152, Université d'Evry Val d'Essonne; INSERM U781, Université Paris Descartes, Hôpital Necker, Paris; Rhumatologie A, Hôpital Cochin, APHP, Université Paris Descartes, Paris; Médecine Interne, Université Lille II, Lille; INSERM EMI-U 00-10, CHU Bretonneau, Tours; Service de Rhumatologie, Hôpital Hautepierre, Université Louis Pasteur, Strasbourg; Médecine Interne, Hôpital Cochin, APHP, Paris Descartes Université, Paris; Service de Médecine Interne, Hôpital Saint-Antoine, APHP, Université Pierre et Marie Curie, Paris; Service de Médecine Interne 2, Hôpital Pitié-Salpêtrière, AP-HP, Université Paris 6, Paris; Laboratoire de Pharmacologie, INSERM ESPRI, HP2 EA 3745, Faculté de Médecine de Grenoble, Grenoble; Clinique Universitaire De Médecine Vasculaire, Pôle Pluridisciplinaire de Médecine, Centre Hospitalier Universitaire, Grenoble; and Laboratoire de Biochimie Hormonale et Génétique, Hôpital Ambroise Paré, AP-HP, Université Versailles Saint Quentin Yvelines, Boulogne, France.

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P. Dieudé, MD, PhD; K. Dawidowicz, MD, Service de Rhumatologie, Hôpital Bichat Claude Bernard, APHP; M. Guedj, PhD, Laboratoire Statistique et Génome, UMR CNRS-8071 / INRA-1152, Université d'Evry Val d'Essonne; Y. Legrain, PhD, INSERM U781, Université Paris Descartes, Hôpital Necker; J. Wipff, MD, PhD, INSERM U781, Université Paris Descartes, Hôpital Necker, Rhumatologie A, Hôpital Cochin,

APHP; E. Hachulla, MD, PhD, Professor, Médecine Interne, Université Lille II; E. Diot, MD, PhD, INSERM EMI-U 00-10, CHU Bretonneau; J. Sibilias, MD, PhD, Professor, Université Louis Pasteur, Service de Rhumatologie, Hôpital Hautepierre; L. Mouthon, MD, PhD, Professor, Paris Descartes Université, Médecine Interne, Hôpital Cochin, APHP; J. Cabane, MD, PhD, Professor, Université Pierre et Marie Curie, Service de Médecine Interne, Hôpital Saint-Antoine, APHP; Z. Amoura, MD, PhD, Professor, Université Paris 6, Service de Médecine Interne 2, Hôpital Pitié-Salpêtrière, AP-HP; J.-L. Crakowski, MD, PhD, Laboratoire de Pharmacologie, INSERM ESPRI, HP2 EA 3745, Faculté de Médecine de Grenoble; P. Carpentier, MD, PhD, Clinique Universitaire De Médecine Vasculaire, Pôle Pluridisciplinaire de Médecine, Centre Hospitalier Universitaire; J. Avouac, MD, PhD, INSERM U781, Université Paris Descartes, Hôpital Necker, Rhumatologie A, Hôpital Cochin, APHP; O. Meyer, MD, Professor, Université Diderot-Paris 7, Service de Rhumatologie, Hôpital Bichat Claude Bernard, APHP; A. Kahan, MD, PhD, Professor, Université Paris Descartes, Rhumatologie A, Hôpital Cochin, APHP; C. Boileau, PharmD, PhD, Professor, INSERM U781, Université Paris Descartes, Hôpital Necker, Laboratoire de Biochimie Hormonale et Génétique, Hôpital Ambroise Paré, AP-HP, Université Versailles Saint Quentin Yvelines; Y. Allanore, MD, PhD, Prof, INSERM U781, Université Paris Descartes, Hôpital Necker, Rhumatologie A, Hôpital Cochin, APHP.

Address correspondence to Dr. P. Dieudé, Service de Rhumatologie, Hôpital Bichat Claude Bernard, 46 rue Henri Huchard, 75018 Paris, France. E-mail: philippe.dieude@bch.aphp.fr

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Systemic sclerosis (SSc) is an orphan complex multiorgan disease affecting the microvascular network, the immune system, and connective tissue. Progressive organ failure makes SSc a severe chronic disease leading to major disability or death¹. To date no drug has proven its efficacy to counteract the generalized multiorgan fibrotic process². Recent data have highlighted the poor efficacy of current available drugs, in particular in the 2 major causes of death, interstitial lung disease³ and pulmonary arterial hypertension⁴.

The type 1 interferon (IFN) pathway has been postulated to play a key role in autoimmune diseases⁵. Indeed, an increased expression of type 1 IFN genes by peripheral blood mononuclear cells, referred to as an IFN signature, has been detected in multiple autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and Sjögren's syndrome (SS) and in a subgroup of patients with SSc⁶⁻⁹. The interferon regulatory factors (IRF) are major regulators of genes activated by the type 1 IFN. Recently, the interferon regulatory factor 5 gene (*IRF5*) has been identified as an autoimmune disease susceptibility gene in the background of SS, SLE, RA, and SSc¹⁰⁻¹³. Our original finding of an association between the *IRF5* and SSc provided additional support for the pivotal role of *IRF5* and the type 1 IFN pathway in SSc¹⁴. We found that the functional *IRF5* rs2004640 single-nucleotide polymorphism (SNP), which creates a donor splice site in intron 1 of the *IRF5* gene resulting in transcription of the alternative exon 1B, displayed association with SSc in 2 independent patient cohorts from French Caucasian populations¹⁴. Further, our data provided new clues for a role of *IRF5* and IFN pathways in fibrosing alveolitis. Recently, different additional functional *IRF5* variants have been convincingly identified as autoimmune genetic factors. Among these *IRF5* variants, the rs10954213 SNP alters the polyadenylation site of *IRF5*, the rare A allele leading to a shorter and more stable mRNA¹⁵. A 5-bp biallelic insertion deletion polymorphism (CGGGG indel) located in the first intron of the *IRF5* gene, resulting in increased binding of the transcription factor SP1 to the risk allele of the 5-bp indel, was also reported to confer susceptibility to autoimmunity¹⁶⁻¹⁸. Interestingly, the functional CGGGG indel polymorphism could be unambiguously inferred from haplotype reconstructions due to the high linkage disequilibrium with the 3 following SNPs: rs377385, rs2004640, and rs10954213¹⁷. Therefore, the aim of our study was to investigate whether *IRF5* haplotypes determined by those 3 SNPs may be also involved in the genetic background of SSc and to account for the respective subphenotypes.

MATERIALS AND METHODS

Study population and design. We performed a large case-control association study including 827 SSc patients and 989 controls from the French SSc network as described^{14,19}. All SSc patients were classified according to the cutaneous subtype classification of LeRoy, *et al*²⁰, and phenotypically assessed as recommended²¹. Controls consisted of healthy unrelated indi-

viduals ethnically matched to the SSc cases. All subjects were of white French Caucasian ethnicity, defined by all 4 grandparents being white French Caucasian. The ethics committee of Cochin Hospital, Paris, approved the study and all subjects gave their written informed consent.

As reported, SSc cases with co-occurrence of a known *IRF5*-associated autoimmune disease (SLE and/or SS and/or RA and/or multiple sclerosis) were excluded prior to the analyses¹⁴. As reported, SSc patients with any ground-glass opacity on thoracic high-resolution computed tomography scan were considered to have interstitial lung involvement^{22,23}. Pulmonary vascular and heart involvements were assessed by cardiac echocardiography and right-heart catheterization in cases suspected of having pulmonary arterial hypertension²⁴.

Autoantibody analysis. SSc patients were tested for antinuclear antibodies (ANA) using indirect immunofluorescence (IIF) and HEP-2 cells as antigen substrate (Antibodies Inc., Davis, CA, USA). Specific SSc antibodies were systematically assessed; anticentromere antibodies (ACA) were determined by their distinctive IIF pattern on HEP-2 cells. Anti-topoisomerase I antibodies were determined by counter-immunoelectrophoresis.

Genotyping. The 3 SNPs were genotyped using a competitive allele-specific polymerase chain reaction (PCR) system (Kaspar genotyping, Kbioscience, Hoddeston, UK) and the Taqman SNP genotyping assay-allelic discrimination method (Applied Biosystems, Foster City, CA, USA) as described^{14,19}. *IRF5* rs2004640 was previously genotyped for the same individuals in our cohort²⁵. The average genotype completeness for the 3 SNPs tested was 89% for the SSc and control samples. The accuracy was > 99%, by duplicate genotyping of 10% of all samples. The CGGGG indel polymorphic marker was amplified by fluorescent PCR and genotyped on an ABI-Prism 3100 device (Applied Biosystems, Courtaboeuf, France) and analyzed with Genotyper software in 100 (i.e., 200 chromosomes) randomly selected individuals homozygous for *IRF5* risk haplotype and in 40 (80 chromosomes) individuals homozygous for the protective haplotype.

Statistical analysis. Single-marker and haplotype analyses were performed in the SSc patients and controls having full genotype information for the 3 *IRF5* SNPs (743 SSc patients, 880 controls). Statistical analyses were performed using the R software (version 2.6.0). The level of significance for all tests corresponds to a type I error rate $\alpha = 5\%$. The Bonferroni correction was applied for all the tests for a "hypothesis-generating step" comparing SSc cases and controls (10 phenotypic subsets; *p* values adjusted). All odds ratios are provided with 95% confidence interval. Conformity to Hardy-Weinberg equilibrium was tested using a classical 1 degree of freedom chi-square test in controls. Individual association analyses of *IRF5* SNPs with SSc were performed by comparing cases and controls with Fisher's exact test on genotypes. Corresponding odds ratios were assessed using standard logistic regression analysis with the most frequent genotype taken as the reference. The same procedure was applied in subgroups stratified according to SSc phenotypes. An analysis of haplotype diversity was realized for the 3 SNPs with the expectation-maximization (EM) algorithm as implemented in the haplo.stats R library. Specific *p* values and odds ratios were obtained by comparing each haplotype with the more common haplotype in the population, applying Fisher's exact test.

RESULTS

***IRF5* single-marker analysis.** Genotype frequencies of the 3 *IRF5* SNPs were in Hardy-Weinberg equilibrium in the control population.

Among the 829 patients and 989 controls investigated, complete genotyping data for the 3 *IRF5* variants were available for 743 and 880 individuals, respectively. Allelic and genotypic frequencies of the *IRF5* rs3757385, rs2004640, and rs10954213 SNPs were in good agreement with frequencies reported among the European Caucasian population¹⁷. The case-control association study involving

743 SSc cases and 880 controls revealed an association between *IRF5* rs3757385, rs2004640, and rs10954213 variants and SSc (Table 1). The magnitude of the association was most important for *IRF5* homozygous genotypes for the risk allele of rs3757385 and rs2004640 SNP, suggesting a dosage effect ($p = 0.009$, OR 1.51, 95% CI 1.10–2.08 and $p = 3.5 \times 10^{-5}$, OR 1.81, 95% CI 1.36–2.40, respectively).

We tested whether the associated SNP constituted an independent effect. Pair-wise correlation coefficient (r^2) analysis found rs3757385 in relatively high linkage disequilibrium with rs2004640 ($r^2 = 0.57$) and rs10954213 ($r^2 = 0.56$). Conversely, rs2004640 was not in strong linkage disequilibrium with rs10954213 ($r^2 = 0.34$). Multiple logistic regression analysis of the 3 SNP revealed no independent effect: the rs2004640 was the only variant that remained significantly associated conditionally to the 2 others ($p = 0.0086$).

IRF5 haplotype analysis. Among the 8 *IRF5* haplotypes defined by the 3 *IRF5* rs3757385, rs2004640, and rs10954213 SNP, 4 common haplotypes (frequency > 5% in both controls and cases) were predicted from our sample. Of the 4 haplotypes, 2 were at a frequency of 25% or more in the controls. As previously observed in a European Caucasian population¹⁷, these 2 common haplotypes C-T-A and A-G-G were present at a frequency of 44% and 33%, respectively, in our control chromosomes. Taking into account the *IRF5* risk alleles rs3757385*C, rs2004640*T, and rs10954213*A, we tested for association of the *IRF5* C-T-A risk haplotype (“R” haplotype). The *IRF5* “R” haplotype was associated with SSc as its frequency was significantly increased in SSc patients (49%) compared to controls

(44%) ($p_{\text{adj}} = 0.024$, OR 1.23, 95% CI 1.07–1.40). We also observed that the C-T-A “mirror” haplotype, i.e., the A-G-G haplotype, acts with a protective (“P” haplotype) effect, its frequency being decreased in the SSc population (27.8%) compared to controls (32.9%) ($p_{\text{adj}} = 8.8 \times 10^{-3}$, OR 0.78, 95% CI 0.68–0.90; Table 2).

Phenotype-genotype correlation in SSc. We previously reported results identifying *IRF5* rs2004640 as a new SSc susceptibility gene, with a strong effect in the genetic background of diffuse cutaneous SSc (dcSSc) and SSc-related fibrosing alveolitis¹⁴. Following those results we investigated the effect of the *IRF5* “R” and “P” haplotypes in subsets of SSc [dcSSc, limited cutaneous SSc (lcSSc), and patients with and without fibrosing alveolitis].

In this study, we observed a strong association of the *IRF5* rs2004640 TT genotype with both dcSSc ($p_{\text{adj}} = 2.6 \times 10^{-3}$, OR 2.01, 95% CI 1.39–3.05) and SSc-related pulmonary fibrosis ($p_{\text{adj}} = 3.7 \times 10^{-4}$, OR 2.19, 95% CI 1.50–3.20). The TT risk genotype was also strongly associated with the subset of severe organ involvement, compared to controls ($p_{\text{adj}} = 2.2 \times 10^{-4}$, OR 2.16, 95% CI 1.50–3.09). The *IRF5* rs3757385 variant was also found to be associated with the different SSc subsets; conversely, no association was detected with rs10954213 (Table 1).

However, regarding the phenotype-genotype correlation, intra-cohort analyses failed to detect any significant differences comparing the allele and genotype frequencies of the 3 *IRF5* SNP and the 3 subsets (i.e., cutaneous phenotype, pulmonary fibrosis, and severe organ involvement; data not shown).

Table 1. Genotype and allele frequency comparisons of the *IRF5* rs3757385, rs2004640, and rs10954213 polymorphisms in SSc patients and controls. For each *IRF5* SNP p values are given as follows: risk allele, homozygous and heterozygous genotypes for the risk allele.

	rs3757385					P _{adj} [*]	OR (95% CI)	rs2004640					P _{adj} [*]	OR (95% CI)	rs10954213					P _{adj} [*]	OR (95% CI)
	CC	CA	AA	C	n (%)			TT	TG	GG	T	n (%)			AA	AG	GG	G	n (%)		
SSc,	341	318	84	67.3	0.001	1.27 (1.10–1.47)						3.7 × 10 ^{−5}	1.34 (1.17–1.54)						0.035	1.17	
n = 743	(45.9)	(42.8)	(11.3)		0.009	1.51 (1.10–2.08)	240	369	134	57.1	3.5 × 10 ^{−5}	1.81 (1.36–2.40)	303	345	95	64.0	0.063	(1.01–1.34)			
					0.58		(32.3)	(49.7)	(18.0)		0.008	1.41 (1.09–1.49)	(40.8)	(46.4)	(12.8)				0.50		
					0.44						7.1 × 10 ^{−4}	1.50 (1.23–1.84)							0.21		
dcSSc,	115	106	31	66.7	0.15		98	106	48	59.9	2.6 × 10 ^{−3}	2.01 (1.39–3.05)	103	114	35	63.5	0.34				
n = 252	(45.6)	(42.1)	(12.3)		0.95		(38.9)	(42.1)	(19.0)		0.52		(40.9)	(45.2)	(13.9)		0.97				
					5.9 × 10 ^{−3}	1.35 (1.14–1.60)					8.9 × 10 ^{−3}	1.32 (1.12–1.55)							0.14		
lcSSc,	211	192	45	68.5	0.04	1.75 (1.19–2.56)	135	238	75	56.7	5.1 × 10 ^{−3}	1.82 (1.29–2.55)	187	211	50	65.3	0.18				
n = 448	(47.1)	(42.9)	(10.0)		0.29		(30.1)	(53.1)	(16.8)		0.01	1.62 (1.20–2.20)	(41.7)	(47.1)	(11.2)		0.18				
					5.5 × 10 ^{−3}	1.42 (1.16–1.74)					9.2 × 10 ^{−5}	1.54 (1.27–1.86)							0.44		
With FA,	149	109	34	69.7	0.23		113	127	52	60.5	3.7 × 10 ^{−3}	2.19 (1.50–3.20)	128	124	40	65.1	0.16				
n = 292	(51.0)	(37.3)	(11.6)		0.72		(38.7)	(43.5)	(17.8)		0.23		(43.8)	(42.5)	(13.7)		0.78				
					0.22						0.05	1.27 (1.08–1.51)							0.96		
Non-FA,	173	182	38	66.5	0.33		117	210	70	55.9	0.03	1.69 (1.19–2.39)	157	194	46	63.9	0.69				
n = 397	(43.6)	(45.8)	(10.6)		0.27		(29.5)	(52.9)	(17.6)		0.07		(39.5)	(48.9)	(11.6)		0.19				
Controls,	330	427	123	61.8			221	436	223	49.9			315	433	132	60.4					
n = 880	(37.5)	(48.5)	(14.0)				(25.1)	(49.6)	(25.3)				(35.8)	(49.2)	(15.0)						

* Adjusted after Bonferroni correction for multiple comparisons. dcSSc: diffuse cutaneous systemic sclerosis; lcSSc: limited cutaneous systemic sclerosis; FA: fibrosing alveolitis.

Table 2. *IRF5* haplotype distribution in SSc patients and controls and haplotype-phenotype correlation.

	<i>IRF5</i> Haplotype Frequency, %	P _{adj} *	p**	OR (95% CI)
<i>IRF5</i> C-T-A risk haplotype (R)				
SSc, n = 743	48.9	0.024	0.0081	1.23 (1.07–1.40)
dcSSc, n = 252	50.9	0.037		1.32 (1.10–1.59)
lcSSc, n = 448	43.8	NS		
FA, n = 292	50.6	0.037		1.32 (1.09–1.59)
Non-FA, n = 397	48.4	NS	NS	
Controls, n = 880	43.8			
<i>IRF5</i> A-G-G protective haplotype (P)				
SSc, n = 743	27.8	8.8×10^{-3}	NS	0.78 (0.68–0.90)
dcSSc, n = 252	28.2	NS		
lcSSc, n = 448	32.9	NS		
FA, n = 292	24.1	3.7×10^{-4}		0.64 (0.51–0.79)
Non-FA, n = 397	29.6	NS	0.018	
Controls, n = 880	32.9			

* Adjusted for comparison between SSc patients and controls. ** Comparison between SSc patients with and without a specific phenotype (i.e., dcSSc vs lcSSc, SSc patients with FA vs those without, severe subset vs non-severe subset). NS: not statistically significant; other abbreviations as in Table 1.

Phenotype-haplotype correlation in SSc. Regarding the *IRF5* C-T-A “R” haplotype, we found an association restricted to dcSSc ($p_{\text{adj}} = 0.037$, OR 1.32, 95% CI 1.10–1.59). The *IRF5* A-G-G “P” haplotype was found to act with a protective effect restricted to the diffuse cutaneous subtype and the subset of SSc patients with fibrosing alveolitis (Table 2). When phenotype-haplotype correlation was investigated, intra-cohort analyses revealed a significantly increased frequency of the “R” haplotype in dcSSc compared to lcSSc ($p = 0.0081$). Conversely, the “P” haplotype was found to be underrepresented in the subset of SSc patients having fibrosing alveolitis compared to those without ($p = 0.018$); the frequency of the “R” haplotype did not differ between the 2 SSc subsets (Table 2). As fibrosing alveolitis is reported to be linked to dcSSc, we next compared the frequencies of the *IRF5* “P” haplotype between patients with and those without fibrosing alveolitis in the lcSSc subset: we observed a decreased frequency of the “P” haplotype in the patient subset without fibrosing alveolitis; however, this did not achieve statistical significance (data available upon request from the author).

As previously stated, the susceptibility CGGGG indel allele was inferred from the 2 *IRF5* “R” and “P” haplotypes. Among a group of 100 SSc patients and controls who were homozygous for the “R” haplotype, 100% were carrying the CGGGG insertion allele; conversely, among 40 individuals homozygous for the *IRF5* “P” haplotype, none had the CGGGG indel susceptibility allele, corroborating that the “R” haplotype harbors the CGGGG indel risk allele.

DISCUSSION

We previously reported an association between the functional *IRF5* rs2004640 polymorphism and SSc¹⁴. Identification of *IRF5* as a genetic susceptibility factor shared by various autoimmune diseases is an important step for understanding type I IFN-driven autoimmunity. Further, this has generated new clues about interstitial lung disease in the context of SSc, which has become a leading cause of death, without established therapeutic options. However, to date, many *IRF5* functional variants have been identified as risk factors for different autoimmune diseases and remain to be investigated in SSc. Our study is the first to provide evidence for association between 2 *IRF5* haplotypes and SSc. We identify an *IRF5* risk haplotype (“R”) defined by the alleles rs3757385*C, rs2004640*T, and rs10954213*A (C-T-A) that confers susceptibility to SSc. The *IRF5* “R” haplotype was found to confer susceptibility to defined subsets of SSc including dcSSc and SSc-related pulmonary fibrosis. These findings illustrate that *IRF5* may contribute to the typical heterogeneity of SSc. This observation is in good agreement with our original study¹⁴, as the main sub-phenotype associations were the diffuse cutaneous and fibrosing alveolitis subsets. In this sample, intra-cohort single-marker analyses failed to provide evidence for using one of the 3 *IRF5* variants as a marker for a particular SSc subset. This discrepancy with our previous findings could be related to the cross-sectional design of the study and a smaller sample size (743 SSc cases, 880 controls) leading to a weaker power to detect such intra-cohort associations. Of

interest, phenotype-haplotype correlation analysis did detect intra-cohort association signals. Indeed, *IRF5* “R” and/or “P” haplotypes were able to discriminate dcSSc and fibrosing alveolitis. Those results emphasize that *IRF5* haplotype analyses are more informative than single-marker analyses of rs3757385, rs2004640, and rs10954213, suggesting the haplotype biomarker could be helpful for risk stratification of patients with SSc.

It is noteworthy that the same risk haplotype has been reported to be associated with high serum levels of type 1 IFN in patients with SLE²⁶. It could be hypothesized that the *IRF5* “R” haplotype harbors one or multiple causal polymorphisms. Interestingly, a 5-bp biallelic insertion-deletion polymorphism (CGGGG indel) located in the first intron of the *IRF5* gene was recently reported to be associated with multiple autoimmune diseases¹⁶⁻¹⁸. The CGGGG risk insertion, which results in increased binding of the SP1 transcription factor, has recently been reported to regulate the expression of *IRF5* mRNA^{27,28}. The CGGGG risk insertion suggested to be the causal variant of *IRF5* in SLE and in primary SS could be unambiguously inferred from haplotype reconstructions with the 3 *IRF5* SNP tested in our study¹⁷. Hence, the *IRF5* “R” haplotype is the exclusive haplotype carrying the CGGGG insertion risk allele, suggesting a possible contribution of this functional variant in the genetic background of SSc¹⁷.

As previously reported, the susceptibility CGGGG indel allele was unambiguously inferred from the 2 *IRF5* “R” and “P” haplotypes in a French Caucasian population. This haplotype was also reported to confer susceptibility to multiple sclerosis in the European Caucasian population¹⁷. However, resequencing of the *IRF5* gene in patients homozygous for the “R” haplotype is required for a conclusion that the CGGGG insertion deletion is the definite *IRF5* causal variant. Further, it is notable that multiple *IRF5* SNP including rs2070197 and rs12539741, located in the 3’UTR, were reported to confer susceptibility to SLE and may be involved in SSc susceptibility¹⁵. Of interest, those SNP are in perfect linkage disequilibrium with several additional Hapmap SNP distributed over a 10-kb region located 3’ of the *IRF5* gene that contains the transportin 3 (*TNPO3*) gene. The reported pair-wise linkage disequilibrium between these SNP and the CGGGG indel is very low, suggesting that the SNP could tag an independent SLE signal association; this remains to be investigated in SSc¹⁶.

Genetic studies of SSc susceptibility have made great progress, and considering the current efforts, more genetic risk factors will be identified. In contrast, the field of the genetics of SSc heterogeneity is relatively unexplored. Following our results, *IRF5* should be investigated in genetics of SSc phenotypic heterogeneity. Identification of the *IRF5* gene as a susceptibility factor for multiple autoimmune disorders emphasizes that similar immunogenetic mechanisms underlie autoimmune diseases. It is also impor-

tant for understanding type I IFN-driven autoimmunity — IFN regulatory factors (IRF), such as *IRF5*, coordinate the expression of type I IFN, which are assumed to participate in the pathogenesis of connective tissue disorders including SLE, SS, and SSc^{6,7,9}. *IRF5* also coordinates the expression of interleukin 6²⁹, which was found to be elevated in plasma serum of patients with dcSSc and SSc-related pulmonary fibrosis^{30,31}. Increased understanding of the role of genetics in disease outcomes in SSc may promote the development of new therapies.

Our study provides evidence for *IRF5* as a new SSc gene playing a key role in disease severity, and supports the pivotal role of type 1 IFN in the pathophysiology of SSc. Further research is needed on the pathogenesis of this severe autoimmune disease, for which there is no cure.

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