

PDF hosted at the Radboud Repository of the Radboud University Nijmegen

The following full text is a publisher's version.

For additional information about this publication click this link.

<http://hdl.handle.net/2066/117843>

Please be advised that this information was generated on 2021-09-27 and may be subject to change.

The Systemic Lupus Erythematosus *IRF5* Risk Haplotype Is Associated with Systemic Sclerosis

F. David Carmona^{1,*}, Jose-Ezequiel Martin¹, Lorenzo Beretta², Carmen P. Simeón³, Patricia E. Carreira⁴, José Luis Callejas⁵, Mónica Fernández-Castro⁶, Luis Sáez-Comet⁷, Emma Beltrán⁸, María Teresa Camps⁹, María Victoria Egurbide¹⁰, the Spanish Scleroderma Group¹, Paolo Airó¹¹, Raffaella Scorza², Claudio Lunardi¹², Nicolas Hunzelmann¹³, Gabriela Riemekasten¹⁴, Torsten Witte¹⁵, Alexander Kreuter¹⁶, Jörg H. W. Distler¹⁷, Rajan Madhok¹⁸, Paul Shiels¹⁸, Jacob M. van Laar¹⁹, Carmen Fonseca²⁰, Christopher Denton²⁰, Ariane Herrick²¹, Jane Worthington²¹, Annemie J. Schuerwegh²², Madelon C. Vonk²³, Alexandre E. Voskuyl²⁴, Timothy R. D. J. Radstake^{23,25}, Javier Martín¹

1 Instituto de Parasitología y Biomedicina López-Neyra, CSIC, Granada, Spain, **2** Referral Center for Systemic Autoimmune Diseases, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano, University of Milan, Milan, Italy, **3** Department of Internal Medicine, Hospital Vall d'Hebron, Barcelona, Spain, **4** Department of Rheumatology, Hospital 12 de Octubre, Madrid, Spain, **5** Department of Internal Medicine, Hospital Clínico San Cecilio, Granada, Spain, **6** Department of Rheumatology, Hospital Puerta de Hierro Majadahonda, Madrid, Spain, **7** Department of Internal Medicine, Hospital Universitario Miguel Servet, Zaragoza, Spain, **8** Department of Rheumatology, Hospital General Universitario de Valencia, Valencia, Spain, **9** Department of Internal Medicine, Hospital Carlos Haya, Málaga, Spain, **10** Department of Internal Medicine, Hospital de Cruces, Barakaldo, Spain, **11** Servizio di Reumatologia ed Immunologia Clinica Spedali Civili, Brescia, Italy, **12** Department of Medicine, Università degli Studi di Verona, Verona, Italy, **13** Department of Dermatology, University of Cologne, Cologne, Germany, **14** Department of Rheumatology and Clinical Immunology, Charité University Hospital, Berlin, Germany, **15** Hannover Medical School, Hannover, Germany, **16** Ruhr University of Bochum, Bochum, Germany, **17** Department of Internal Medicine 3, Institute for Clinical Immunology, University of Erlangen-Nuremberg, Erlangen, Germany, **18** Centre for Rheumatic Diseases, Glasgow Royal Infirmary, University of Glasgow, Glasgow, United Kingdom, **19** Institute of Cellular Medicine, Newcastle University, Newcastle, United Kingdom, **20** Centre for Rheumatology, Royal Free and University College Medical School, London, United Kingdom, **21** Arthritis Research UK Epidemiology Unit, University of Manchester, Manchester Academic Health Science Centre, Manchester, United Kingdom, **22** Department of Rheumatology, Leiden University Medical Center, Leiden, The Netherlands, **23** Department of Rheumatology, Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands, **24** Department of Rheumatology, VU University Medical Center, Amsterdam, The Netherlands, **25** Department of Rheumatology and Clinical Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

Abstract

Systemic sclerosis (SSc) is a fibrotic autoimmune disease in which the genetic component plays an important role. One of the strongest SSc association signals outside the human leukocyte antigen (HLA) region corresponds to interferon (IFN) regulatory factor 5 (*IRF5*), a major regulator of the type I IFN pathway. In this study we aimed to evaluate whether three different haplotypic blocks within this *locus*, which have been shown to alter the protein function influencing systemic lupus erythematosus (SLE) susceptibility, are involved in SSc susceptibility and clinical phenotypes. For that purpose, we genotyped one representative single-nucleotide polymorphism (SNP) of each block (rs10488631, rs2004640, and rs4728142) in a total of 3,361 SSc patients and 4,012 unaffected controls of Caucasian origin from Spain, Germany, The Netherlands, Italy and United Kingdom. A meta-analysis of the allele frequencies was performed to analyse the overall effect of these *IRF5* genetic variants on SSc. Allelic combination and dependency tests were also carried out. The three SNPs showed strong associations with the global disease (rs4728142: $P = 1.34 \times 10^{-8}$, OR = 1.22, CI 95% = 1.14–1.30; rs2004640: $P = 4.60 \times 10^{-7}$, OR = 0.84, CI 95% = 0.78–0.90; rs10488631: $P = 7.53 \times 10^{-20}$, OR = 1.63, CI 95% = 1.47–1.81). However, the association of rs2004640 with SSc was not independent of rs4728142 (conditioned $P = 0.598$). The haplotype containing the risk alleles (rs4728142*A-rs2004640*T-rs10488631*C: $P = 9.04 \times 10^{-22}$, OR = 1.75, CI 95% = 1.56–1.97) better explained the observed association (likelihood P -value = 1.48×10^{-4}), suggesting an additive effect of the three haplotypic blocks. No statistical significance was observed in the comparisons amongst SSc patients with and without the main clinical characteristics. Our data clearly indicate that the SLE risk haplotype also influences SSc predisposition, and that this association is not sub-phenotype-specific.

Citation: Carmona FD, Martin J-E, Beretta L, Simeón CP, Carreira PE, et al. (2013) The Systemic Lupus Erythematosus *IRF5* Risk Haplotype Is Associated with Systemic Sclerosis. PLoS ONE 8(1): e54419. doi:10.1371/journal.pone.0054419

Editor: Masataka Kuwana, Keio University School of Medicine, Japan

Received: September 5, 2012; **Accepted:** December 11, 2012; **Published:** January 23, 2013

Copyright: © 2013 Carmona et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by the following grants: JM was funded by GEN-FER from the Spanish Society of Rheumatology, SAF2009-11110 from the Spanish Ministry of Science, CTS-4977, and CTS-180 from Junta de Andalucía, RETICS Program, RD08/0075 (RIER) from Instituto de Salud Carlos III (ISCIII), Spain, within the VI PN de I+D+i 2008–2011 (FEDER), and is sponsored by the Orphan Disease Program grant from the European League Against Rheumatism (EULAR). This study was also funded by PI-0590-2010, from Consejería de Salud y Bienestar Social, Junta de Andalucía, Spain. NH and JM are funded by Consejería de Salud, Junta de Andalucía, through PI-0590-2010. FDC was supported by Consejo Superior de Investigaciones Científicas (CSIC) through the program JAE-DOC. TRDJR was funded by the VIDI laureate from the Dutch Association of Research (NWO) and Dutch Arthritis Foundation (National Reumafonds). TW was granted by DFG WI 1031/6.1. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: dcarmona@ipb.csic.es

† These authors contributed equally to this work.

‡ Membership of the Spanish Scleroderma Group is provided in File S1.

Introduction

Systemic sclerosis (SSc) is a chronic multisystem connective tissue disorder characterized by fibrotic events, vascular damage and autoantibody production. Two main clinical subtypes have been defined based on the extent of skin involvement, limited cutaneous scleroderma (lcSSc) and diffuse cutaneous scleroderma (dcSSc) [1]. Recent candidate gene and genome-wide association studies (GWASs) clearly suggest that an important genetic component underlies this disease. In this regard, an increasing number of *loci* have been reported to be convincingly associated with the susceptibility and clinical manifestations of SSc in the last years. However, the causal functional mutations responsible for these associations have not been unambiguously identified yet in most cases [2].

Outside the HLA region, interferon (IFN) pathway genes, which encode cytokines with critical modulatory effects on innate and adaptive immunity, have been shown to represent a key component of the genetic network leading to autoimmune processes. Interestingly, a misregulated expression of type I IFN genes, also referred to as IFN signature, have been observed in peripheral white blood cells patient subsets of several autoimmune diseases [3,4,5,6], thus suggesting that the IFN signaling plays a crucial role in autoimmunity. Indeed, multiple single-nucleotide polymorphisms (SNPs) of the IFN regulatory factor 5 gene (*IRF5*), a major regulator of the type I IFN induction, have been associated with different rheumatic disorders such as SSc, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and Sjögren's syndrome (SS) [7,8,9,10]. The *IRF5* association with SLE was narrowed down to three different haplotype blocks that seem to have independent functional consequences, including 1) alteration of the protein stability, 2) creation of a donor splice site in intron 1 resulting in transcription of an alternative exon 1B, and 3) modification of the 3'UTR length which affects expression levels [11]. Subsequent studies in SSc patients suggested that genetic variation within *IRF5* correlate with SSc severity and survival [12,13].

Based on the above, we decided to explore whether the functional haplotype blocks described by Graham *et al.* [11] were also susceptibility signals affecting SSc development and progression. For that purpose, we analysed the allele frequencies of three representative *IRF5* genetic variants that have been previously associated with SSc [8,14] in five large Caucasian European cohorts and performed allelic combination and dependency tests.

Patients and Methods

Study Population

We recruited a total of 3,361 SSc patients and 4,012 unaffected controls of Caucasian origin from five different European countries, including an initial cohort from Spain and four replication cohorts from Germany, The Netherlands, Italy and United Kingdom. Case and control sets were matched by geographical origin and ethnicity. Written informed consent from all participants and approval from the local ethical committees of all centres involved in the study were obtained in accordance with the tenets of the Declaration of Helsinki. All SSc patients fulfilled the classification criteria by Leroy *et al.* [15]. The clinical features

of the different SSc cohorts are shown in **Table 1**. Case sets were further subdivided based on their skin involvement into limited cutaneous scleroderma (lcSSc) and diffuse cutaneous scleroderma (dcSSc) subgroups [15], and by autoantibody status according to the presence/absence of anti-centromere antibodies (ACA) or anti-topoisomerase antibodies (ATA), which were detected using standard procedures. Pulmonary fibrosis (PF) was diagnosed by high resolution computed tomography (HRCT).

SNPs Selection and Genotyping

Samples were genotyped for three *IRF5* tag SNPs, rs10488631, rs2004640, and rs4728142, representative of three different haplotype blocks (refers to as Groups 1–3, respectively) which have been reported to have functional roles in SLE patients [11]: Group 1 includes SNPs tagging a 30-bp in-frame INDEL variant of exon 6 that alters protein stability; Group 2 includes an exon 1B splice site variant; and Group 3 corresponds to genetic variants located in a conserved polyadenylation signal sequence that alters the length of the 3'UTR, thus affecting expression levels.

Genomic DNA was obtained from peripheral blood cells using standard procedures, and genotyping was performed using TaqMan® 5' allele discrimination assays (IDs: C__2691242_10, C__9491614_10, and C__2691222_10), in a 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, California, USA).

Statistical Analysis

The statistical power of the study was calculated with Power Calculator for Genetic Studies 2006 software (<http://www.sph.umich.edu/csg/abecasis/CaTS/reference.html>), which implements the methods described in Skol *et al.* [16].

Table 1. Main clinical features of systemic sclerosis patients included in the study.

| Feature | N (%) | | | | |
|---------|--------------|-------------|-------------|-------------|-------------|
| | Spain | Germany | Netherlands | Italy | UK |
| Female | 1089 (89.14) | 494 (85.83) | 323 (81.83) | 644 (91.50) | 388 (83.33) |
| Male | 133 (10.86) | 81 (14.17) | 72 (18.17) | 60 (8.50) | 77 (16.67) |
| lcSSc | 843 (68.99) | 338 (58.78) | 271 (68.61) | 515 (73.15) | 336 (72.26) |
| dcSSc | 379 (31.01) | 237 (41.22) | 124 (31.39) | 189 (26.85) | 129 (27.74) |
| ACA+ | 560 (45.83) | 214 (37.22) | 99 (25.06) | 312 (44.32) | 169 (36.34) |
| ACA- | 617 (50.49) | 341 (59.30) | 291 (73.67) | 385 (54.69) | 285 (61.29) |
| ATA+ | 267 (21.85) | 174 (30.26) | 106 (26.84) | 238 (33.81) | 71 (15.27) |
| ATA- | 881 (72.09) | 376 (65.39) | 284 (71.90) | 461 (65.48) | 382 (82.15) |
| PF+ | 294 (24.06) | 175 (30.43) | 144 (36.46) | 205 (29.12) | 111 (23.87) |
| PF- | 830 (67.92) | 327 (56.87) | 158 (40.00) | 400 (56.82) | 189 (40.65) |

Data are referred to the total analysed individuals.

ACA; anti-centromere antibodies; ATA, anti-topoisomerase antibodies; dcSSc, diffuse cutaneous systemic sclerosis; lcSSc, limited cutaneous systemic sclerosis; PF, pulmonary fibrosis.

doi:10.1371/journal.pone.0054419.t001

PLINK v1.07 (<http://pngu.mgh.harvard.edu/purcell/plink/>) [17] was used to carry out all statistical analyses of allele frequencies. *P*-values were obtained by performing 2×2 contingency tables and χ^2 test and/or Fisher's exact test, when appropriate. Since the association between *IRF5* and SSc has been confirmed in several independent studies [2], we considered appropriate to set the significance threshold at *P* = 0.05. Odds ratios (OR) and 95% confidence intervals were calculated according to Woolf's method. Breslow–Day (BD) test method was used to estimate the homogeneity amongst populations. Pooled analyses were performed by Mantel-Haenszel test under fixed effects, or DerSimonian-Laird if the BD test reached statistical significance.

Dependency of association between the studied genetic variants was determined by conditional logistic regression analysis as implemented in PLINK, and the allelic combinations were tested using PLINK, StatsDirect (V.2.6.6; StatsDirect, Altrincham, UK), and Hapview (V.4.2) [18].

To analyse whether allelic combinations would better explain the possible association than the genetic variants independently, we compared the goodness of fit of both models using PLINK. For that purpose, we calculated the deviance (defined as $-2 \times$ the log likelihood), which follows a χ^2 distribution, to assess the significance of the improvement in fit. If statistically significant differences in the improvement of fit were observed when the haplotype effect was considered, we assumed that this model was more informative explaining the putative association.

Results

The overall statistical power of the study, based on previous *IRF5* reports, to detect associations with OR = 1.2 at 0.05 significance level was 100% for rs2004640 and rs4728142, and 93% for rs10488631 (**Table S1 in File S1**). Additionally, no significant departure from Hardy-Weinberg equilibrium was observed either in cases or controls in each analysed population (*P* = 0.05).

Allele Test

The results of the global analyses of the discovery cohort and the four independent replication populations separately are shown in **Table S2 in File S1**. Since the Breslow-Day test evidenced no heterogeneity of the ORs amongst the different cohorts (*P* = 0.05), a combined meta-analysis was performed to test the overall effect of the *IRF5* genetic variants in the whole dataset (**Table 2**). The pooled analysis showed that the three SNPs were strongly associated with the global disease (rs4728142: *P* = 1.34×10^{-8} , OR = 1.22, CI 95% = 1.14–1.30; rs2004640: *P* = 4.60×10^{-7} , OR = 0.84, CI 95% = 0.78–0.90; rs10488631: *P* = 7.53×10^{-20} , OR = 1.63, CI 95% = 1.47–1.81). Highly significant *P*-values were also yielded when the different phenotype subgroups were compared against the control population (**Table S3 in File S1**). However, no statistical significance was observed in the comparisons amongst SSc patients accordingly with the presence/absence of the different clinical characteristics and autoantibody profile (**Table 2**), i.e. lcSSc vs. dcSSc (rs4728142: *P* = 0.564; rs2004640: *P* = 0.971; rs10488631: *P* = 0.086), SSc ACA+ vs. SSc ACA- (rs4728142: *P* = 0.359; rs2004640: *P* = 0.357; rs10488631: *P* = 0.449), SSc ATA+ vs. SSc ATA- (rs4728142: *P* = 0.154; rs2004640: *P* = 0.128; rs10488631: *P* = 0.259), and SSc PF+ vs. SSc PF- (rs4728142: *P* = 0.934; rs2004640: *P* = 0.397; rs10488631: *P* = 0.945), thus indicating the three *IRF5* polymorphisms are indeed associated with the global disease and there was no phenotype-specific association.

Conditional Logistic Regression

We decided to perform pairwise conditioning analyses to test whether there could be any dependency amongst them (**Table 3**). The analysis showed that every SNP maintained its statistical significance after conditioning to the other two except for rs2004640, which was dependent of rs4728142. The moderate linkage disequilibrium between them ($r^2 \sim 0.68$) could explain this fact (**Table S4 in File S1**).

Haplotype Analysis

We also analysed the allelic combinations of the *IRF5* genetic variants included in this study according to the global disease (**Table 4**). The most associated haplotype was that containing the risk alleles of the three SNPs (rs4728142*A-rs2004640*T-rs10488631*C: *P* = 9.04×10^{-22} , OR = 1.75, CI 95% = 1.56–1.97). The protective haplotype also showed a very significant *P*-value (rs4728142*G-rs2004640*G-rs10488631*T: *P* = 2.48×10^{-7} , OR = 0.84, CI 95% = 0.78–0.89).

When comparing the haplotype model with the independent SNP model, we observed a statistically significant improvement of the goodness of fit compared to rs4728142 (likelihood *P*-value = 1.23×10^{-17}), rs2004640 (likelihood *P*-value = 1.94×10^{-19}), or rs10488631 (likelihood *P*-value = 1.48×10^{-4}) individually.

On the other hand, we also performed a sub-phenotype analysis of allelic combinations to test whether some haplotype could influence a specific clinical condition (**Table S5 in File S1**). This analysis only showed a residual *P*-value for a low frequency haplotype in the PF+/PF- comparison (rs4728142*G-rs2004640*T-rs10488631*T: *P* = 0.041, OR = 1.28, CI 95% = 1.02–1.61). The rest of allelic combinations did not reach statistical significance in any other comparison.

Discussion

GWAS data have confirmed *IRF5* as one of the strongest associated signals with SSc [19,20]. In addition, it has been proposed that particular *IRF5* functional genetic elements contribute to SLE pathophysiology through their relationship with auto-antibodies and IFN α production [21,22]. These data indicate that this gene may represent a crucial member of the genetic component underlying this type of autoimmune diseases [23].

Previous published data suggested that two different *IRF5* haplotypes influence specific SSc phenotypes. It was hypothesised that these haplotypes may explain a possible *IRF5* association with dcSSc and PF, likely by tagging an intronic 5-bp biallelic insertion-deletion polymorphism (INDEL), which would represent the real causal functional variant [12]. However, our results are not in agreement with this idea, since we did not find evidence for a specific genetic association between *IRF5* and any of the major clinical manifestations, despite the fact that two out of the three genetic variants comprising the previously described risk haplotype were covered in our analysis (rs2004640 and rs10954213 that is highly correlated with rs4728142). A similar discrepancy was also observed by Sarif *et al.* [13], who failed to replicate the *IRF5* haplotype effect on PF described by Dieudé *et al.* [12]. Our data are, however, consistent with recent GWAS follow-up studies that did not show a phenotype-specific association of *IRF5* with SSc, but a clear association with the overall disease [14,24]. It should be noted that one of the SNPs included in this study, rs4728142, has been shown to correlate with longer survival and a milder pulmonary involvement in SSc patients [13]. Taking this together with our results, it could be speculated that, although the risk variants of *IRF5* do not predispose to develop PF, they may

Table 2. Meta-analysis of *IRF5* genetic variants accordingly with the global disease and the presence or absence of the main clinical features.

| SNP | Position | 1/2 | Subgroup (N) | Genotype, N (%) | | | MAF (%) | M-H allele test | | | | | |
|---------------|--------------|-------------|---------------------|-----------------|--------------|--------------|---------|-----------------|--------------------------|------------------------------|--------|------------------|------|
| | | | | 1/1 | 1/2 | 2/2 | | P-value | OR [CI 95%] [†] | P _{BD} [‡] | | | |
| rs4728142 | 3'UTR | A/G | Controls (n = 3933) | 787 (20.01) | 1924 (48.92) | 1222 (31.07) | 44.47 | 1.34E-08 | 1.22 [1.14–1.30] | 0.32 | | | |
| | | | SSc (n = 3128) | 769 (24.58) | 1549 (49.52) | 810 (25.90) | 49.34 | | | | | | |
| | | | lcSSc (n = 2142) | 533 (24.88) | 1059 (49.44) | 550 (25.68) | 49.60 | | | | 0.564 | 0.97 [0.87–1.08] | 0.61 |
| | | | dcSSc (n = 986) | 236 (23.94) | 490 (49.70) | 260 (26.37) | 48.78 | | | | | | |
| | | | ACA- (n = 1781) | 444 (24.93) | 852 (47.84) | 485 (27.23) | 48.85 | | | | 0.359 | 1.05 [0.95–1.16] | 0.08 |
| | | | ACA+ (n = 1268) | 307 (24.21) | 658 (51.89) | 303 (23.90) | 50.16 | | | | | | |
| | | | ATA- (n = 2222) | 531 (23.90) | 1110 (49.95) | 581 (26.15) | 48.87 | | | | 0.154 | 1.09 [0.97–1.22] | 0.28 |
| | | | ATA+ (n = 793) | 215 (27.11) | 378 (47.67) | 200 (25.22) | 50.95 | | | | | | |
| | | | PF- (n = 1797) | 427 (23.76) | 929 (51.70) | 441 (24.54) | 49.61 | | | | 0.934 | 1.00 [0.89–1.12] | 0.97 |
| PF+ (n = 893) | 237 (26.54) | 409 (45.80) | 247 (27.66) | 49.44 | | | | | | | | | |
| rs2004640 | Exon 1 | G/T | Controls (n = 3912) | 897 (22.93) | 1895 (48.44) | 1120 (28.63) | 47.15 | 4.60E-07 | 0.84 [0.78–0.90] | 0.12 | | | |
| | | | SSc (n = 3122) | 584 (18.71) | 1511 (48.40) | 1027 (32.90) | 42.91 | | | | | | |
| | | | lcSSc (n = 2143) | 406 (18.95) | 1026 (47.88) | 711 (33.18) | 42.88 | | | | 0.971 | 1.00 [0.90–1.12] | 0.23 |
| | | | dcSSc (n = 979) | 178 (18.18) | 485 (49.54) | 316 (32.28) | 42.95 | | | | | | |
| | | | ACA- (n = 1772) | 354 (19.98) | 833 (47.01) | 585 (33.01) | 43.48 | | | | 0.357* | 0.91 [0.74–1.12] | 0.01 |
| | | | ACA+ (n = 1272) | 211 (16.59) | 643 (50.55) | 418 (32.86) | 41.86 | | | | | | |
| | | | ATA- (n = 2216) | 424 (19.13) | 1076 (48.56) | 716 (32.31) | 43.41 | | | | 0.128 | 0.91 [0.81–1.03] | 0.43 |
| | | | ATA+ (n = 793) | 137 (17.28) | 377 (47.54) | 279 (35.18) | 41.05 | | | | | | |
| | | | PF- (n = 1800) | 327 (18.17) | 899 (49.94) | 574 (31.89) | 43.14 | | | | 0.397 | 0.95 [0.85–1.07] | 0.84 |
| PF+ (n = 883) | 163 (18.46) | 417 (47.23) | 303 (34.31) | 42.07 | | | | | | | | | |
| rs10488631 | INDEL tagger | C/T | Controls (n = 3958) | 41 (1.04) | 625 (15.79) | 3292 (83.17) | 8.93 | 7.53E-20 | 1.63 [1.47–1.81] | 0.11 | | | |
| | | | SSc (n = 3148) | 70 (2.22) | 749 (23.79) | 2329 (73.98) | 14.12 | | | | | | |
| | | | lcSSc (n = 2165) | 46 (2.12) | 494 (22.82) | 1625 (75.06) | 13.53 | | | | 0.086 | 1.14 [0.98–1.33] | 0.96 |
| | | | dcSSc (n = 983) | 24 (2.44) | 255 (25.94) | 704 (71.62) | 15.41 | | | | | | |
| | | | ACA- (n = 1798) | 48 (2.67) | 425 (23.64) | 1325 (73.69) | 14.49 | | | | 0.449 | 0.94 [0.81–1.10] | 0.92 |
| | | | ACA+ (n = 1273) | 21 (1.65) | 306 (24.04) | 946 (74.31) | 13.67 | | | | | | |
| | | | ATA- (n = 2236) | 48 (2.15) | 508 (22.72) | 1680 (75.13) | 13.51 | | | | 0.259* | 1.17 [0.89–1.54] | 0.03 |
| | | | ATA+ (n = 799) | 21 (2.63) | 212 (26.53) | 566 (70.84) | 15.89 | | | | | | |
| | | | PF- (n = 1818) | 43 (2.37) | 425 (23.38) | 1350 (74.26) | 14.05 | | | | 0.945 | 0.99 [0.84–1.17] | 0.94 |
| PF+ (n = 886) | 19 (2.14) | 213 (24.04) | 654 (73.81) | 14.16 | | | | | | | | | |

[†]Odds ratio for the minor allele.

^{*}Breslow-Day P-value.

[‡]DerSimonian-Laird random effects model P-value. SSc, systemic sclerosis; lcSSc, limited cutaneous SSc; dcSSc, diffuse cutaneous SSc; ACA, anti-centromere antibodies; ATA, anti-topoisomerase antibodies. M-H, Mantel-Haenszel test under fixed effect.

doi:10.1371/journal.pone.0054419.t002

influence the severity of some clinical features like PF. In any case, it is important to note that whereas antibody profile and disease subtypes are clearly a dichotomous outcome, PF can range from

mild-stable to severe-progressive involvement (and the utilised approach for definition of PF does not differentiate the different severity scales of this disease manifestation) [25].

Table 3. Conditional logistic regression analysis for the *IRF5* polymorphisms considering the five populations as covariate.

| SNP | P-value | P-value: add to rs10488631 | rs10488631 P-value: add to SNP | P-value: add to rs2004640 | rs2004640 P-value: add to SNP |
|------------|------------------|-------------------------------|-----------------------------------|------------------------------|----------------------------------|
| rs4728142 | 1.344E-08 | 2.57E-04 | 8.92E-16 | 2.24E-03 | 0.598 |
| rs2004640 | 4.603E-07 | 0.020 | 1.72E-16 | NA | NA |
| rs10488631 | 7.528E-20 | NA | NA | 1.72E-16 | 0.020 |

LD, linkage disequilibrium; SNP, single-nucleotide polymorphism.

doi:10.1371/journal.pone.0054419.t003

Table 4. Pooled-analysis of different allelic combinations of the *IRF5* genomic region according to disease.

| Allelic combination | Freq. Controls | Freq. Cases | P-value | OR [95% CI] |
|---------------------|----------------|-------------|-----------------|------------------|
| GGT | 0.4612 | 0.4181 | 2.48E-07 | 0.84 [0.78–0.89] |
| ATT | 0.3593 | 0.3652 | 0.533 | 1.02 [0.95–1.10] |
| ATC | 0.0731 | 0.1215 | 9.04E-22 | 1.75 [1.56–1.97] |
| GTT | 0.0777 | 0.0652 | 4.47E-03 | 0.83 [0.72–0.94] |
| GTC | 0.0155 | 0.0187 | 0.153 | 1.21 [0.93–1.57] |

Order of the SNPs: rs4728142*A/G | rs2004640*G/T | rs10488631*C/T.

OR, odds ratio.

doi:10.1371/journal.pone.0054419.t004

As stated before, the tag SNPs analysed here are representative of three haplotype blocks that have been reported to affect the function of the protein in different ways, including production of an alternative spliced isoform, alteration of polyadenylation sites that leads to a shorter messenger RNA, and reduction of protein stability [11]. These three polymorphisms showed strong association signals in our study, supported by a high statistical power. Therefore, the functional alterations caused by their risk alleles may be also of high relevance in the pathogenic mechanisms that lead to SSc. However, the rs2004640 signal was dependant of that from rs4728142 in our study cohort. Hence, rs2004640 might not be an independent SSc susceptibility *locus* although it is functionally relevant, which suggests that not all functional variants in a determined risk gene may play an independent role in the associated disease. In any case, as described in SLE [11], we observed a significant additive effect amongst the three analysed SNPs because the haplotypes containing both the risk and protective alleles better explained the association between this *locus* and SSc. Hence, although no functional studies have been carried out yet to unmask the possible implication of the *IRF5* risk alleles in the SSc pathophysiology, it is likely that each one of the protein alterations described above influence the development of SSc individually, and that carrying all the three risk alleles results in a critically reduced protein function that highly increases SSc susceptibility.

References

- Agarwal SK, Reveille JD (2010) The genetics of scleroderma (systemic sclerosis). *Curr Opin Rheumatol* 22: 133–138.
- Martin JE, Bossini-Castillo L, Martin J (2012) Unraveling the genetic component of systemic sclerosis. *Hum Genet* 131: 1023–1037.
- Higgs BW, Liu Z, White B, Zhu W, White WI, et al. (2011) Patients with systemic lupus erythematosus, myositis, rheumatoid arthritis and scleroderma share activation of a common type I interferon pathway. *Ann Rheum Dis* 70: 2029–2036.
- Sozzani S, Bosio D, Scarsi M, Tincani A (2010) Type I interferons in systemic autoimmunity. *Autoimmunity* 43: 196–203.
- Assassi S, Mayes MD, Arnett FC, Gourh P, Agarwal SK, et al. (2010) Systemic sclerosis and lupus: points in an interferon-mediated continuum. *Arthritis Rheum* 62: 589–598.
- Milano A, Pendergrass SA, Sargent JL, George LK, McCalmont TH, et al. (2008) Molecular subsets in the gene expression signatures of scleroderma skin. *PLoS One* 3: e2696.
- Graham RR, Kozyrev SV, Baechler EC, Reddy MV, Plenge RM, et al. (2006) A common haplotype of interferon regulatory factor 5 (IRF5) regulates splicing and expression and is associated with increased risk of systemic lupus erythematosus. *Nat Genet* 38: 550–555.
- Dieudé P, Guedj M, Wipff J, Avouac J, Fajardy I, et al. (2009) Association between the IRF5 rs2004640 functional polymorphism and systemic sclerosis: a new perspective for pulmonary fibrosis. *Arthritis Rheum* 60: 225–233.
- Han SW, Lee WK, Kwon KT, Lee BK, Nam EJ, et al. (2009) Association of polymorphisms in interferon regulatory factor 5 gene with rheumatoid arthritis: a meta-analysis. *J Rheumatol* 36: 693–697.
- Miceli-Richard C, Comets E, Loiseau P, Puechal X, Hachulla E, et al. (2007) Association of an IRF5 gene functional polymorphism with Sjögren's syndrome. *Arthritis Rheum* 56: 3989–3994.
- Graham RR, Kyogoku C, Sigurdsson S, Vlasova IA, Davies LR, et al. (2007) Three functional variants of IFN regulatory factor 5 (IRF5) define risk and protective haplotypes for human lupus. *Proc Natl Acad Sci U S A* 104: 6758–6763.
- Dieudé P, Dawidowicz K, Guedj M, Legrain Y, Wipff J, et al. (2010) Phenotype-haplotype correlation of IRF5 in systemic sclerosis: role of 2 haplotypes in disease severity. *J Rheumatol* 37: 987–992.
- Sharif R, Mayes MD, Tan FK, Gorlova OY, Hummers LK, et al. (2012) IRF5 polymorphism predicts prognosis in patients with systemic sclerosis. *Ann Rheum Dis* 71: 1197–1202.
- Martin JE, Broen JC, Carmona FD, Teruel M, Simeon CP, et al. (2012) Identification of CSK as a systemic sclerosis genetic risk factor through Genome Wide Association Study follow-up. *Hum Mol Genet* 21: 2825–2835.
- LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, et al. (1988) Scleroderma (systemic sclerosis): classification, subsets and pathogenesis. *J Rheumatol* 15: 202–205.
- Skol AD, Scott LJ, Abecasis GR, Boehnke M (2006) Joint analysis is more efficient than replication-based analysis for two-stage genome-wide association studies. *Nat Genet* 38: 209–213.
- Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, et al. (2007) PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 81: 559–575.

In conclusion, this study clearly shows that a haplotype of three different functional genetic variants within the *IRF5* region confer susceptibility to SSc. The fact that this association is shared with SLE adds another piece of evidence to the common genetic background of both diseases, and provides a new perspective for the study of the type I IFN pathway and its implication in the development of autoimmune conditions.

Supporting Information

File S1. Table S1. Overall statistical power of the study for each analysed *IRF5* genetic variant at the 5% significance level. **Table S2.** Independent analyses of *IRF5* genetic variants in Caucasian SSc patients and unaffected controls from Europe. **Table S3.** Meta-analysis of *IRF5* genetic variants comparing the main clinical phenotypes with unaffected controls. **Table S4.** Linkage disequilibrium structure of the *IRF5* region analysed in this study. **Table S5.** Pooled-analysis of different allelic combinations of the *IRF5* genomic region according to the presence/absence of specific clinical phenotypes. (DOC)

Acknowledgments

The authors thank Sofia Vargas, Sonia García, and Gema Robledo (from Instituto de Parasitología y Biomedicina 'López-Neyra', CSIC, Spain) for their excellent technical assistance, and all the patients and healthy controls for kindly accepting their essential collaboration. Banco Nacional de ADN (University of Salamanca, Spain) is thanked for supplying the Spanish controls.

Author Contributions

Collection of data: LB CPS PEC JLC MFC LSC EB MTC MVE PA RS CL NH GR TW AK JHWD RM PS JML CF CD AH JW AJS MCV AEV TRDJR. Analysis and interpretation of data: FDC JEM JM LB CPS PEC JLC MFC LSC EB MTC MVE PA RS CL NH GR TW AK JHWD RM PS JML CF CD AH JW AJS MCV AEV TRDJR. Critical revision of the manuscript draft: JEM JM LB CPS PEC JLC MFC LSC EB MTC MVE PA RS CL NH GR TW AK JHWD RM PS JML CF CD AH JW AJS MCV AEV TRDJR. Conceived and designed the experiments: FDC JEM JM. Performed the experiments: FDC JEM. Analyzed the data: FDC JEM. Contributed reagents/materials/analysis tools: LB CPS PEC JLC MFC LSC EB MTC MVE PA RS CL NH GR TW AK JHWD RM PS JML CF CD AH JW AJS MCV AEV TRDJR. Wrote the paper: FDC.

18. Barrett JC, Fry B, Maller J, Daly MJ (2005) Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 21: 263–265.
19. Allanore Y, Saad M, Dieude P, Avouac J, Distler JH, et al. (2011) Genome-wide scan identifies TNIP1, PSORS1C1, and RHOB as novel risk loci for systemic sclerosis. *PLoS Genet* 7: e1002091.
20. Radstake TR, Gorlova O, Rueda B, Martin JE, Alizadeh BZ, et al. (2010) Genome-wide association study of systemic sclerosis identifies CD247 as a new susceptibility locus. *Nat Genet* 42: 426–429.
21. Niewold TB, Kelly JA, Kariuki SN, Franek BS, Kumar AA, et al. (2012) IRF5 haplotypes demonstrate diverse serological associations which predict serum interferon alpha activity and explain the majority of the genetic association with systemic lupus erythematosus. *Ann Rheum Dis* 71: 463–468.
22. Niewold TB, Kelly JA, Flesch MH, Espinoza LR, Harley JB, et al. (2008) Association of the IRF5 risk haplotype with high serum interferon-alpha activity in systemic lupus erythematosus patients. *Arthritis Rheum* 58: 2481–2487.
23. Kozyrev SV, Alarcon-Riquelme ME (2007) The genetics and biology of Irf5-mediated signaling in lupus. *Autoimmunity* 40: 591–601.
24. Gorlova O, Martin JE, Rueda B, Kocleman BP, Ying J, et al. (2011) Identification of novel genetic markers associated with clinical phenotypes of systemic sclerosis through a genome-wide association strategy. *PLoS Genet* 7: e1002178.
25. Gabrielli A, Avvedimento EV, Krieg T (2009) Scleroderma. *N Engl J Med* 360: 1989–2003.