Confirmation of TNIP1 but not RHOB and PSORS1C1 as systemic sclerosis risk factors in a large independent replication study

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INTRODUCTION

Systemic sclerosis or scleroderma (SSc) is a complex autoimmune disorder that affects the connective tissue causing fibrosis in the skin and different internal organs. The contribution of different genetic factors to the development and prognosis of the disease is now widely accepted. Over the past few years, genome-wide association studies (GWAS) have been a useful tool in the genetic dissection of autoimmune pathologies and other complex diseases. Radstake et al performed the first SSc GWAS in Caucasian populations, which represented the first large-scale GWAS in SSc. This work reinforced the association within the HLA region, especially with the HLA-DQB1 gene, which was also reported in a comprehensive multiethnic SSc HLA LA study. It also confirmed the associations found in STAT4 and IRF5 and identified CD247 as a new SSc risk locus. It is worth mentioning that the role of CD247 in SSc has recently been independently replicated. This GWAS has led to three follow-up studies, which have described several novel SSc susceptibility factors, ie, IRF5, GRB10, SOX5, NOTCH4, IL12RB2, CSK, PSD3 and NFKB1. Interestingly, SOX5 and NOTCH4 are directly related to the fibrotic process, which is a main hallmark of SSc.

A GWAS has recently been performed in a French Caucasian SSc discovery cohort. In this GWAS, 17 single-nucleotide polymorphisms (SNP) showing tier two associations were selected for follow-up in independent cohorts. Three of the selected SNP were located within the HLA region corresponding to the HLA-DQB1 and PSORS1C1 genes; and the remaining SNP were located in six independent non-HLA loci. After the replication step, the associations of HLA-DQB1, CD247, STAT4 and IRF5 were confirmed, and six SNP located in three loci (TNIP1, RHOB, PSORS1C1) were proposed as novel SSc risk factors.

It has been observed that associations identified from a single GWAS, even passing the established statistical significance thresholds, tend to have different internal locations. The contribution of different genetic factors to the development and prognosis of the disease is now widely accepted. Over the past few years, genome-wide association studies (GWAS) have been a useful tool in the genetic dissection of autoimmune pathologies and other complex diseases. Radstake et al performed the first SSc GWAS in Caucasian populations, which represented the first large-scale GWAS in SSc. This work reinforced the association within the HLA region, especially with the HLA-DQB1 gene, which was also reported in a comprehensive multiethnic SSc HLA LA study. It also confirmed the associations found in STAT4 and IRF5 and identified CD247 as a new SSc risk locus. It is worth mentioning that the role of CD247 in SSc has recently been independently replicated. This GWAS has led to three follow-up studies, which have described several novel SSc susceptibility factors, ie, IRF5, GRB10, SOX5, NOTCH4, IL12RB2, CSK, PSD3 and NFKB1. Interestingly, SOX5 and NOTCH4 are directly related to the fibrotic process, which is a main hallmark of SSc.

A GWAS has recently been performed in a French Caucasian SSc discovery cohort. In this GWAS, 17 single-nucleotide polymorphisms (SNP) showing tier two associations were selected for follow-up in independent cohorts. Three of the selected SNP were located within the HLA region corresponding to the HLA-DQB1 and PSORS1C1 genes; and the remaining SNP were located in six independent non-HLA loci. After the replication step, the associations of HLA-DQB1, CD247, STAT4 and IRF5 were confirmed, and six SNP located in three loci (TNIP1, RHOB, PSORS1C1) were proposed as novel SSc risk factors.

It has been observed that associations identified from a single GWAS, even passing the established statistical significance thresholds, tend to have
inflated effect sizes. This effect size is called the winner’s curse, and it also affects the predictive ability of the discovered associations and the estimate of the risk variance explained by the associations. Replication in independent comparable populations is thus essential for firmly establishing a genotype-phenotype association. Therefore, we aimed to perform a large-scale replication study of the novel SSC genetic risk factors identified by GWAS strategy in an independent white European and US SSC population.

**PATIENTS AND METHODS**

**Subjects**

4389 SSC patients and 7611 controls of Caucasian ancestry (Spain, The Netherlands, USA, Italy, Sweden, UK and Norway) were included in this study. Patients were classified as having limited or diffuse SSC, as defined by LeRoy et al. The following clinical data were collected for ascertainment of the clinical phenotype of the patients with SSC: age, gender, disease duration and presence of SSC-associated autoantibodies, antitopoisomerase (ATA) and anti-centromere (ACA).

Supplementary table S1 (available online only) shows the cohort-specific SSC patient data. The control population consisted of unrelated healthy individuals recruited in the same geographical regions as SSC patients and matched by age, sex and ethnicity with the SSC patient groups. Local ethics committees from all the participating centres approved the study. Both patients and controls were included in the study after written informed consent.

In the meta-analysis with previously published data by Allanore et al., which includes 2246 SSC patients and 5702 healthy controls from France, Italy, Germany and Eastern Europe, the total cohort size reached 6635 patients and 13,313 healthy controls from France, Italy, Germany, and Eastern Europe (Spain, The Netherlands, USA, Italy, Sweden, UK and Norway) were analysed using TaqMan SNP genotyping assays in a 7900HT Real-Time PCR System from Applied Biosystems following the manufacturer’s suggestions (Applied Biosystems, Foster City, California, USA). The differences in the number of samples included in the analyses of each polymorphism correspond to the availability of the genotype data in each platform (see supplementary table S2, available online only). Stringent quality control filters and principal component analysis were applied to the GWAS and the HLA imputed data as described in Radstake et al. The genotyping call-rate for the individuals genotyped using TaqMan assays reached: rs432070 95.77%, rs13021401 94.98%, rs2253287 96.08%, rs4958881 90.75%, rs3792783 93.29% and rs3150573 95.19%.

**Statistical analysis**

PLINK (V1.07) software (http://pngu.mgh.harvard.edu/purcell/plink/) was used for individual population association tests (significance was calculated by 2χ^2 contingency tables and Fisher’s exact test or χ^2 when necessary, and in the case of the haplotypes each haplotype was tested against all others), logistic regression and conditional logistic regression analyses. The different cohorts were considered covariates in the logistic regression analyses. OR and their 95% CI were reported. TNIP1 haplotypes were constructed using PLINK (V1.07) and HaploView 4.2 (http://www.broadinstitute.org/haploview/haploview) only with those individuals successfully genotyped for the three included variants (2432 SSCs and 3496 healthy controls). The Breslow–Day test was performed as implemented in PLINK and StatsDirect to assess the homogeneity of the association among populations. Pooled analyses and meta-analyses were carried out using a Mantel–Haenszel test under a fixed effects by PLINK (V1.07), METAL (http://www.sph.umich.edu/csg/abecasis/metal/) and StatsDirect (V2.6.6) in the case of haplotypes. Significant heterogeneity among populations was found in the meta-analysis of RHOB locus polymorphisms; consequently, in this case a random effects model was applied using StatsDirect. Genotypic frequency distributions for the meta-analysis were kindly provided by Allanore et al. for the meta-analysis by personal communication. All cohorts were in Hardy–Weinberg equilibrium at a significance level of 0.01 for all the included SNP. Power was calculated using the software Power Calculator for Genetic Studies 2006 and assuming an additive model at the 5% significance level and previously reported OR (rs342070 minor allele A frequency (MAF) 0.226, OR 1.20; rs13021401 MAF 0.225, OR 1.21; rs2253287 MAF 0.196, OR 1.31; rs4958881 MAF 0.115, OR 1.29; rs3792783 MAF 0.152, OR 1.29; rs3150573 MAF 0.321, OR 1.25).
As shown in table 1, none of the tested polymorphisms in RHOB, TNIP1, and PSORS1C1 variant with all the independent signals from the HLA region with SSc is influenced primary by the autoantibody profile of the patients.\(^5\)\(^7\) We aimed to test for an uncov-
ered primary association of the HLA genes. We thus carried out a step-wise logistic regression conditional analysis of the analysed PSORS1C1 variant with all the independent signals from the most significant to the lowest observed p values in the HLA region. The considered conditions included SNPs, imputed aminoacids positions and imputed classic HLA-alleles (as described in Raychaudhuri et al\(^6\)\(^7\) in the analysed GWAS cohorts (Spain I, The Netherlands I and USA I) (unpublished data). Standard logistic regression analyses in the GWAS cohorts showed evidence of association only in the whole disease versus controls and in the ACA-positive patients versus controls comparisons (\(P_{\text{log}}=0.054, OR=1.09; P_{\text{log}}=0.01, OR=1.12\), respectively). However, the previously mentioned association with the whole set of SSc patients lost its significance when it was conditioned to the HLA-DBF1*1301 allele (p value

### Table 1  Pooled analysis of the novel SSc non-HLA susceptibility loci

<table>
<thead>
<tr>
<th>CHR</th>
<th>BP</th>
<th>SNP</th>
<th>Locus</th>
<th>1/2</th>
<th>Subgroup (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>20548952</td>
<td>rs342070</td>
<td>RHOB</td>
<td>C/T</td>
<td>Controls (n=7193)</td>
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<td>448 (6.23)</td>
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<td>2713 (37.72)</td>
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<td>4032 (56.05)</td>
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<td>2609 (35.09)</td>
</tr>
<tr>
<td>2</td>
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<td>rs13021401</td>
<td>RHOB</td>
<td>C/T</td>
<td>Controls (n=6557)</td>
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<td>405 (6.18)</td>
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<td>2416 (36.85)</td>
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<td>3736 (56.98)</td>
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<td>3226 (48.60)</td>
</tr>
<tr>
<td>5</td>
<td>150420290</td>
<td>rs2233287</td>
<td>TNIP1</td>
<td>C/T</td>
<td>Controls (n=7164)</td>
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<td>1226 (17.11)</td>
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<td>491 (56.31)</td>
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<td>446 (25.57)</td>
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<td>C/T</td>
<td>Controls (n=7126)</td>
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<td>131 (1.82)</td>
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<td>5539 (77.12)</td>
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<td>C/T</td>
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<td>40 (3.84)</td>
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<td>326 (41.32)</td>
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<td>675 (84.64)</td>
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<td>609 (85.90)</td>
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</table>

**Controls are used as reference for all comparisons.**

*All p values have been calculated for the allelic model.

ACA+, anti-centromere autoantibody-positive patients; ATA+, anti-topoisomerase autoantibody-positive patients; BP, base pair; CHR, chromosome; CTRL, healthy controls; MAF, minor allele (A) frequency; \(p_{\text{Mantel}}\), Mantel–Haenszel test under fixed effect; \(p_{\text{log}}\), homogeneity Breslow-Day test; 1/2, minor allele/major allele; SNP, single nucleotide polymorphism.

**PSORS1C1 analysis**

The study of the PSORS1C1 reported variant, rs5130573, showed a suggestive but heterogeneous association of this polymorphism with increased SSc susceptibility (table 2). Moreover, the association was maintained in all the subgroups (including the ACA-negative and ATA-negative subsets) except for the ACA-positive patients. Considering that the association of the HLA region with SSc is influenced primarily by the autoantibody profile of the patients,\(^5\)\(^7\) we aimed to test for an uncov-
ered influence of the HLA genes. We thus carried out a step-wise logistic regression conditional analysis of the analysed PSORS1C1 variant with all the independent signals from the most significant to the lowest observed p values in the HLA region. The considered conditions included SNPs, imputed aminoacids positions and imputed classic HLA-alleles (as described in Raychaudhuri et al\(^6\)\(^7\) in the analysed GWAS cohorts (Spain I, The Netherlands I and USA I) (unpublished data). Standard logistic regression analyses in the GWAS cohorts showed evidence of association only in the whole disease versus controls and in the ACA-positive patients versus controls comparisons (\(P_{\text{log}}=0.054, OR=1.09; P_{\text{log}}=0.01, OR=1.12\), respectively). However, the previously mentioned association with the whole set of SSc patients lost its significance when it was conditioned to the HLA-DBF1*1301 allele (p value

\(P_{\text{random}}=0.19\); \(P_{\text{random}}=0.15\).
Figure 1  (A) Forest plot for the meta-analysis of the rs2233287 TNIP1 genetic variant. (B) Forest plot for the meta-analysis of the rs4958881 TNIP1 genetic variant. (C) Forest plot for the meta-analysis of the rs3792783 TNIP1 genetic variant.
In this study we conducted a large multicentre replication of the novel SSC risk variants identified by Allanore et al., and we confirmed the association of the TNIP1 locus with SSC. However, the associations observed in the RHOB gene and the independence of PSORS1C1 from the HLA region were not supported by our data. 

Due to the lack of association observed in the RHOB locus, we suggest that the initial association reported in this gene might have been a false positive finding. It is worth mentioning that RHOB has never been robustly associated with an autoimmune disease, and the previously reported association with SSC in this gene did not reach the GWAS significance level even after replication.

PSORS1C1 was proposed as an HLA-independent SSC risk factor. The authors performed a dependence analysis controlling for the described association in the HLA-DQB1 gene and reported the independence of both loci. Nevertheless, HLA-DQB1 has been specifically related with the ACA-positive subset of patients, and both in our data and in the previous study no association of PSORS1C1 with ACA positivity has been shown. Therefore, a deeper analysis of this locus was needed, and we performed for the first time conditional logistic regression including all the independent signals in the HLA region. In our initial approach we found a signal in the PSORS1C1 gene that was comparable to the one described in the previous work, however, a comprehensive analysis showed that the PSORS1C1 association is dependent from the HLA-DQB1*01:01, HLA-DQA1*05:01 and HLA-DRB1*11:04 alleles (especially the HLA-DQA1*0501). These HLA loci have previously been reported as ATA-positive risk factors, which is consistent with the lack of association in the ACA-positive subgroup.

Altogether, our data do not confirm PSORS1C1 as an independent player in the SSC genetic susceptibility network. 

Regarding the association of TNFAIP3 interacting protein 1 (TNIP1), our data clearly support TNIP1 as a SSC risk factor. Our replication study confirmed that the association of the three SNPs tested is maintained in all the subsets, indicating that this association peak corresponds to the whole disease. Therefore, TNIP1 might be implicated in the development of the disease but may not act as a disease modifier. Remarkably, TNIP1 is involved in HIV replication, acts as a negative regulator of the nuclear factor κB pathway (a key regulator of the immune response, which has also recently been associated with SSC), and also represses agonist-bound retinoic acid receptors and peroxisome proliferator-activated receptors. Furthermore, recent studies focused on the control of TNIP1 transcription have reported a complex mechanism behind TNIP1 expression that combines constitutive transcription factors and inducible factors (nuclear factor κB and peroxisome proliferator-activated receptors). Interestingly, Allanore et al. showed that the transcription and expression of TNIP1 is decreased both in the skin of SSC patients and SSC cultured fibroblasts, thus the anti-inflammatory effect of this molecule may be reduced in SSC patients, providing evidence for a relevant role of TNIP1 in the disease. TNIP1 is also a well-established risk factor for different autoimmune diseases, such as psoriasis, psoriatic arthritis and systemic lupus erythematosus. Furthermore, TNIP1 association with psoriasis has also been reported in Asian populations, suggesting that the role of TNIP1 in autoimmune diseases is consistent through different ethnicities. Therefore, this locus can be considered a common autoimmune disease risk factor that can be used as a new therapeutic target.

To conclude, our replication study has reinforced the influence of TNIP1 in an increased susceptibility to SSC and its role as a new player in the autoimmune genetic background. Future research will identify the causal variant for the TNIP1 association and its implication in SSC pathophysiology.

**DISCUSSION**

**Table 2** Pooled-analysis of the rs3130573 PSORS1C1 HLA-region genetic variant

| CHR | BP | SNP | 1/2 Subgroup (N) | Genotype, N (%) | MAF N (%) | Allele test | PBM* | OR (95% CI) | PB0
|---|---|---|---|---|---|---|---|---|---|
| 6 | 31214247 | rs3130573 | G/A | Controls (n=7139) | 892 (12.49) 3220 (45.10) 3027 (42.40) | 5004 (35.05) | 1.17E-05 1.14 (1.07 to 1.21) 0.02 | 0.02
|   |   |   |   | SSc (n=4130) | 574 (13.90) 1953 (47.29) 1603 (38.81) | 3101 (37.54) | 0.28 1.05 (0.96 to 1.14) 0.12 | 0.12
|   |   |   |   | dcSSc (n=1187) | 177 (14.91) 561 (47.28) 488 (37.83) | 915 (38.54) | 1.01E-07 1.21 (1.13 to 1.29) 0.05 | 0.05
|   |   |   |   | ACA+ (n=645) | 123 (14.56) 411 (48.64) 311 (36.80) | 657 (38.88) | 1.17E-05 1.14 (1.07 to 1.21) 0.02 | 0.02
|   |   |   |   | ATA+ (n=3147) | 413 (13.92) 1389 (47.00) 1160 (39.08) | 2221 (37.42) | 1.18E-04 1.13 (1.07 to 1.21) 0.03 | 0.03

Controls are used as reference for all comparisons.

*All p values have been calculated for the allelic model.

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conditioning on DPB1*15:01=0.06; OR conditioning on DPB1*15:01=1.08). HLA-DRB1*11:04 (p value conditioning on DRB1*11:04=0.08; OR conditioning on DRB1*11:04=1.07) and the HLA-DQA1*05:01 (p value conditioning on DQA1*05:01=0.36; OR conditioning on DQA1*05:01=1.04) alleles. Moreover, the association with the ACA-negative subset of patients was also shown to be dependent on the HLA-DQA1*05:01 allele (p value conditioning on DQA1*05:01=0.27; OR add to DQA1*05:01=1.05). Therefore, our data suggest that the association of PSORS1C1 with SSC is not independent from the HLA region.
Competing interests None.

Patient consent Obtained.

Ethics approval Local ethics committees from all the participating centres approved the study.

Provenance and peer review Not commissioned; externally peer reviewed.

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