EXTENDED REPORT

Influence of *TYK2* in systemic sclerosis susceptibility: a new *locus* in the IL-12 pathway

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ABSTRACT

Objectives *TYK2* is a common genetic risk factor for several autoimmune diseases. This gene encodes a protein kinase involved in interleukin 12 (IL-12) pathway, which is a well-known player in the pathogenesis of systemic sclerosis (SSc). Therefore, we aimed to assess the possible role of this *locus* in SSc.

Methods This study comprised a total of 7103 patients with SSc and 12 220 healthy controls of European ancestry from Spain, USA, Germany, the Netherlands, Italy and the UK. Four *TYK2* single-nucleotide polymorphisms (V362F (rs2304256), P1104A (rs34536443), I684S (rs12720356) and A928V (rs35018800)) were selected for follow-up based on the results of an Immunochip screening phase of the locus. Association and dependence analyses were performed by the means of logistic regression and conditional logistic regression. Meta-analyses were performed using the inverse variance method.

Results Genome-wide significance level was reached for *TYK2* V362F common variant in our pooled analysis ($p=3.08\times10^{-13}$, OR=0.83), while the association of P1104A, A928V and I684S rare and low-frequency missense variants remained significant with nominal signals ($p=2.28\times10^{-3}$, OR=0.80; $p=1.27\times10^{-3}$, OR=0.59; $p=2.63\times10^{-5}$, OR=0.83, respectively). Interestingly, dependence and allelic combination analyses showed that the strong association observed for V362F with SSc, corresponded to a synthetic association dependent on the effect of the three previously mentioned *TYK2* missense variants.

Conclusions We report for the first time the association of *TYK2* with SSc and reinforce the relevance of the IL-12 pathway in SSc pathophysiology.

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INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune disease that involves extensive fibrosis in the skin and different internal organs, abnormalities of the vascular system and immune imbalance with autoantibody production, particularly anticentromere autoantibodies (ACA) and antitopoisomerase autoantibodies (ATA). The aetiology of the disease is largely unknown, although both environmental and genetic factors are thought to be involved in the disease development.[1]

Large genetic studies, including genome-wide association studies and Immunochip analysis, have identified several immune-related loci underlying the susceptibility to SSc onset.[2 3] Although great advances have been made over the last 7 years, our knowledge of SSc genetic background is still limited, and the numbers of convincingly SSc genetic markers only account for a small proportion of the total genetic variance for the disease.[4 5] Thus, further genetic studies will help to better understand the pathogenic processes implicated in SSc development.

A recent fine-mapping genetic study of a common autoimmunity locus, *TYK2-ICAM*, in rheumatoid arthritis (RA) identified three *TYK2* protein-coding variants as the most likely causal variants responsible for the signal of association in the region. The authors also extended the results into other autoimmune phenotypes, such as systemic lupus erythematosus (SLE), and observed that the three variants are missense mutations predicted to be damaging using functional prediction tools.[6]

TYK2 encodes a tyrosine kinase member of the JAK-STAT family, and mediates signalling of different interleukin 12 (IL-12) family cytokines, such us IL-12 and IL-23. Several polymorphisms in this locus have been associated with other autoimmune diseases, such as psoriasis, multiple sclerosis, Crohn's disease and ulcerative colitis.[7]

Interestingly, SSc Immunochip study[3] found suggestive, but not significant, evidence of association in *TYK2* region (p values ranging from 5×10^{-4} to 5×10^{-2}). Moreover, different functional and genetic studies highlighted the special relevance of IL-12/STAT4 pathway in the disease pathophysiology.[3 4 8 9] Thus, we performed a follow-up study to further investigate whether variations within this genomic region, including

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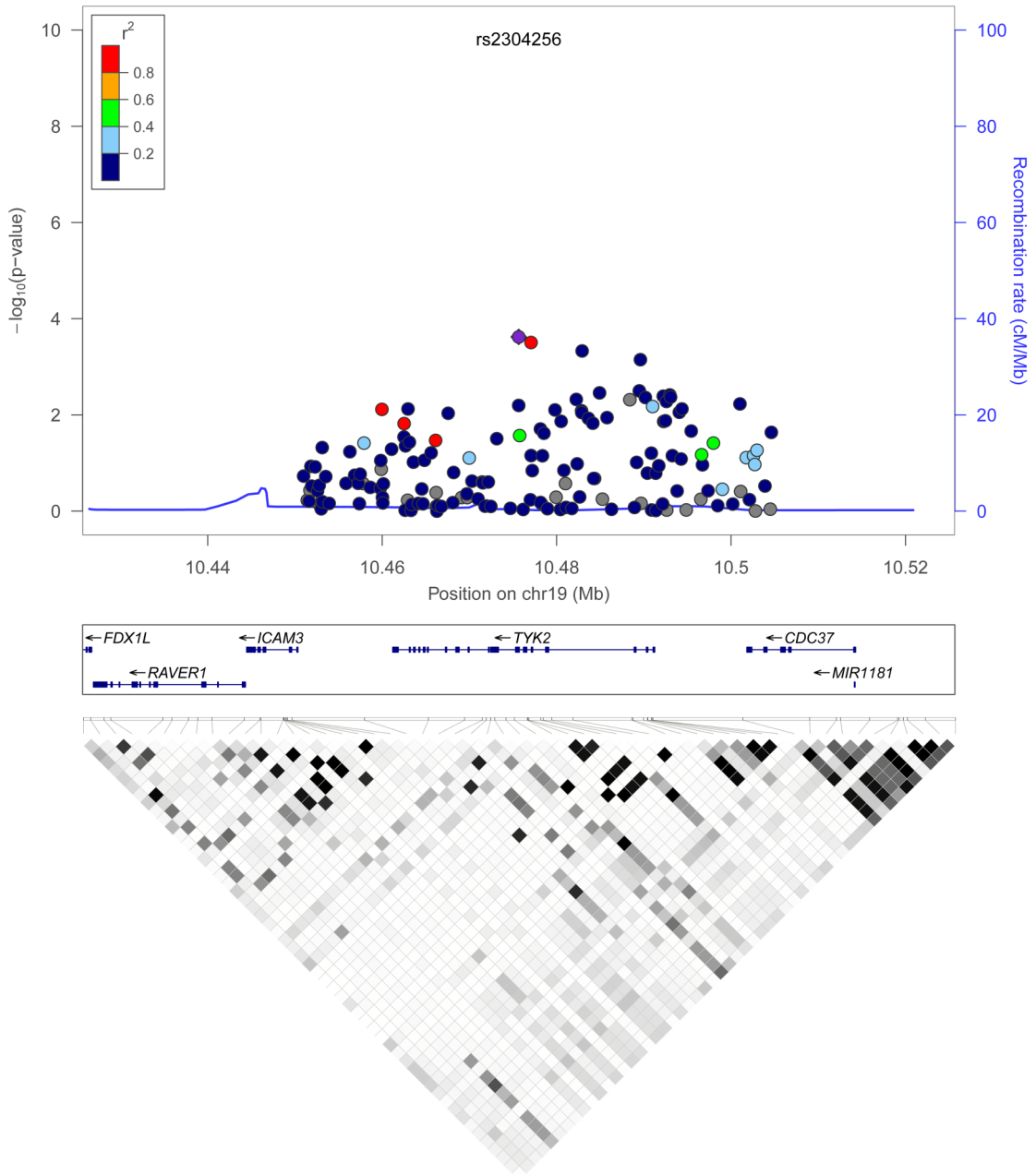

Figure 1 Association result plot for *TYK2* region in the Immunochip screening phase. The p values for association ($-\log_{10}$ values) of each single-nucleotide polymorphism are plotted against their physical position on chromosome 19. The lower panel shows the linkage disequilibrium pattern at the TYK2 *locus* (r^2 values are indicated by colour gradient).

the three variants responsible for the association in RA and other autoimmune phenotypes, are also involved in SSc susceptibility.

METHODS

Study population

This study comprised a total of 7103 patients with SSc and 12 220 healthy controls of European ancestry. The 2118 patients with SSc and 4742 healthy controls from Spain and USA enrolled in the SSc Immunochip screening phase were obtained from the previously published SSc Immunochip study[3] and additional Immunochip data for Spanish patients with SSc and healthy controls. The validation cohort included 4985 SSc cases and 7478 controls from independent case–control sets of European ancestry (Germany, the Netherlands, Italy, UK and USA).

Patients with SSc fulfilled the 1980 American College of Rheumatology classification criteria for this disease or the criteria proposed by LeRoy and Medsger for early SSc.[10 11] In addition, patients were classified as having limited cutaneous SSc or diffuse cutaneous SSc as described in LeRoy *et al*.[12] Patients were also subdivided by autoantibody status according to the presence of ACA or ATA.

Approval from the local ethics committees and written informed consent from all participants were obtained in accordance with the tenets of the Declaration of Helsinki.

Study design

SSc Immunochip screening phase

An initial evaluation of *TYK2* region was performed in the SSc Immunochip screening phase. We included 30 kpb spanning the complete *TYK2* gene and 10 kpb upstream and downstream

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Table 1 Inverse variance meta-analysis of four *TYK2* SNPs in seven different cohorts of patients with SSc and healthy controls (7103 patients with SSc and 12 220 controls)

Chr	SNP	Minor/major	Comment	MAF cases	MAF controls	Inverse variance test		
						p Value	OR (95% CI)*	Q
19	rs34536443 (P1104A)	C/G	missense Pro >Ala	0.023	0.026	2.28E-03	0.80 (0.69 to 0.92)	0.13
19	rs35018800 (A928V)	A/G	missense Ala >Val	0.004	0.008	1.27E-03	0.59 (0.42 to 0.81)	0.34
19	rs12720356 (I684S)	C/A	missense Ile >Ser	0.067	0.078	2.63E-05	0.83 (0.78 to 0.91)	0.27
19	rs2304256 (V362F)	A/C	missense Val >Phe	0.246	0.279	3.08E-13	0.83 (0.79 to 0.87)	0.69

*OR for the minor allele.
Chr, chromosome; MAF, minor allele frequency; Q, heterogeneity value; SNP, single-nucleotide polymorphism; SSc, systemic sclerosis.

from this locus, from base pair 10 450 993 to 10 504 616 in chromosome 19. The analysed genetic region comprised the linkage disequilibrium (LD) block that completely covers *TYK2* (figure 1). Quality control filters and principal component analysis were applied as described in ref. 3. We performed single-nucleotide polymorphism (SNP) genotype imputation of the *TYK2* region as implemented in IMPUTE2 with the use of the 1000 Genomes Phase 1 reference panel.[13 14] After imputation, genotyping data for 154 SNPs were available.

Follow-up phase

Four *TYK2* missense mutations were selected for validation in independent replication cohorts: one common coding variant (V362F (rs2304256)), two low-frequency coding variants (P1104A (rs34536443), I684S (rs12720356)) and one rare coding variant (A928V (rs35018800)). Finally, we performed meta-analysis for the selected SNPs combining the cohorts from both stages.

Genotyping methods

The genotyping of the SSc cases included in the validation cohorts was performed with both TaqMan SNP genotyping technology and Immunochip platform. For TaqMan genotyping system, we used TaqMan 5′ allele discrimination predesigned assays from Applied Biosystems in a LightCycler 480 Real-Time PCR System (Roche Applied Science, Mannheim, Germany). Genotyping call rate was >95% for all the SNPs. The Immunochip genotyping was performed on the Illumina iScan system, as per Illumina protocols, in the Centre for Genomics and Oncological Research (GENYO, Granada, Spain). Control genotyping data partially overlapped with those from previous Immunochip reports.[15–23] If any of the four selected SNPs was missing in a dataset, imputation was applied. Genotype imputation was performed with IMPUTE2 using the 1000 Genomes Phase 1 reference panel.[13 14] The correspondence between Immunochip (including imputed data) and TaqMan genotyping data was >98% for all the SNPs.

Data analysis

Associations of the SNPs with SSc were evaluated by logistic regression analysis in all the cohorts separately. Meta-analysis was performed with inverse-variance weighting under a fixed-effects model as implemented in PLINK V.1.07 software.[24] The combined analysis, including the two phases of the study, was also performed using the inverse variance method based on population-specific logistic regression analyses. p Values <0.05 were considered statistically significant in the association analyses. Heterogeneity between the datasets was assessed using Cochran's Q test. Q values <0.05 were considered statistically significant. Hardy–Weinberg equilibrium (HWE) was tested for all the validation cohorts (HWE p values <0.01 were considered to show significant deviation from the equilibrium). None of the included control cohorts showed significant deviation from HWE for all the genotyped SNPs.

To test the independence of association between each SNP, we performed conditional logistic regression analyses as implemented in PLINK. To analyse the possible effect of A928V (rs35018800) in conditioning analysis, a generalised null linear model including population origin and two variants (P1104A (rs34536443) and I684S (rs12720356)) as covariates was compared against an alternative model including the same variables and A928V (rs35018800) variant by the means of a likelihood ratio test in R. We also assessed the different allelic combinations using PLINK. Allelic combinations with a frequency <0.5% were excluded from the analysis.

Regional association plot for *TYK2* region was performed using LocusZoom V.1.1 software (http://csg.sph.umich.edu/locuszoom/).[25] The HapMap Project phase I, II and III (CEU populations) was used to define the LD pattern across *TYK2* region, and Haploview V.4.2 software (http://www.

Table 2 Dependence analysis by pairwise conditioning of four *TYK2* SNPs in the overall combined cohort (7103 patients with SSc and 12 220 controls)

SNP	MAF cases/controls	Unconditioned p value	OR	*p Value: add to rs2304256	OR† add to rs2304256	*p Value: add to rs34536443	OR† add to rs34536443	*p Value: add to rs35018800	OR† add to rs35018800	*p Value: add to rs12720356	OR† add to rs12720356
rs34536443	0.023/0.026	2.28E-03	0.80	0.02	0.84	NA	NA	1.51E-03	0.79	1.04E-03	0.78
rs35018800	0.004/0.008	1.27E-03	0.59	9.56E-03	0.65	1.11E-03	0.57	NA	NA	1.20E-03	0.59
rs12720356	0.067/0.078	2.63E-05	0.83	0.176	0.94	5.09E-06	0.82	2.22E-05	0.83	NA	NA
rs2304256	0.246/0.279	3.08E-13	0.83	NA	NA	1.37E-05	0.89	4.75E-12	0.84	1.63E-07	0.86

*Single locus test p value when SNP conditioned on rs2304256/rs34536443/rs35018800/rs12720356.
†Single locus test OR when SNP conditioned on rs2304256/rs34536443/rs35018800/rs12720356.
MAF, minor allele frequency; NA, not applicable; SNP, single-nucleotide polymorphism; SSc, systemic sclerosis.

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Table 3 Conditional logistic regression analysis of four *TYK2* SNPs in the overall combined cohort (7103 patients with SSc and 12 220 controls)

			Conditioned to rs2304256, rs35018800, rs12720356		Conditioned to rs2304256, rs12720356, rs34536443		Conditioned to rs2304256, rs35018800, rs34536443		Conditioned to rs34536443, rs12720356		Conditioned to rs35018800, rs12720356, rs34536443	
SNP	Unconditioned p value	OR	p Value	OR	p Value	OR	p Value	OR	p Value	OR	p Value	OR
rs34536443	2.28E-03	0.80	6.94E-04	0.76	NA	NA	NA	NA	NA	NA	NA	NA
rs35018800	1.27E-03	0.59	NA	NA	8.60E-04	0.56	NA	NA	9.39E-04	0.57	NA	NA
rs12720356	2.63E-05	0.83	NA	NA	NA	NA	4.70E-04	0.84	NA	NA	NA	NA
rs2304256	3.08E-13	0.83	NA	NA	NA	NA	NA	NA	0.091	0.94	0.270	0.97

NA, not applicable; SNP, single-nucleotide polymorphism; SSc, systemic sclerosis.

broadinstitute.org/haploview/haploview) was used to perform the LD plot. The statistical power of the combined analysis is shown in online supplementary table S1, and was calculated according to Power Calculator for Genetic Studies 2006 software under an additive model.[26]

RESULTS

SSc Immunochip initial screening

The initial screening of *TYK2* region performed in the SSc Immunochip study showed several tier-two association signals at this locus (figure 1). A common protein-coding missense variant previously associated with SLE showed the strongest association with the disease (V362F (rs2304256) p value=2.39×10^{-4}, OR=0.85).[27–29] This variant and the three *TYK2* protein-coding variants responsible for the association with RA and SLE according to Diogo *et al*[6] were selected for follow-up in independent validation cohorts to confirm the suggestive evidence of association found in this locus with SSc.

Follow-up phase and meta-analysis

Pooled analysis, including the five validation cohorts, revealed significant associations for the four *TYK2* SNPs with SSc at $p<0.05$ (see online supplementary table S2). The meta-analysis combining both steps showed that *TYK2* V362F (rs2304256) variant achieved the genome-wide significance level ($p=3.08\times10^{-13}$, OR=0.83), while P1104A (rs34536443), A928V (rs35018800) and I684S (rs12720356) remained with significant nominal p values ($p=2.28\times10^{-3}$, OR=0.80; $p=1.27\times10^{-3}$, OR=0.59; $p=2.63\times10^{-5}$, OR=0.83, respectively) (table 1). No significant heterogeneity in the ORs among the seven cohorts was observed. The analyses carried out for the main SSc clinical features revealed that the observed association signal rely on the whole disease (data not shown).

Dependence analyses

We then assessed the independence of associations by conditional logistic regression analyses. Although pairwise conditioning results were not conclusive (table 2), the V362F genome-wide significance association was lost when adding the allelic dosage for rs3453644, rs35018800 and rs12720356 as covariates ($p_{cond}=0.270$) (table 3), supporting that the *TYK2* V362F association was dependent on the three rare and low-frequency missense variants. Although A928V (rs35018800) seemed not to exert an effect on V362F (rs2304256) association, model fitting test showed that the regression model, including this rare variant as covariate had a significantly better likelihood than the model excluding it ($p=1.15\times10^{-4}$). Allelic combination tests also confirmed that the V362F association was driven by the presence of the minor alleles of P1104A, A928V and I684S *TYK2* variants, since no genome-wide significant p value was observed for the allelic model carrying only the minor allele of V362F (see online supplementary table S3).

DISCUSSION

The overall analysis of our study reported genome-wide significance level of association for *TYK2* with SSc, providing robust evidence for the implication of this new locus in SSc development.

The meta-analysis showed strong association for V362F common variant, whereas the rare and low-frequency variants—P1104A, A928V and I684S—remained with significant nominal association signals. Although our study was underpowered to detect associations at the genome-wide level of significance for these three missense variants, dependence analyses clearly supported that V362F association was a spurious signal, driven by P1104A, A928V and I684S. This effect is probably due to the high D values between V362F and the three rare and low-frequency variants.

Our findings are in accordance with the results reported by Diogo *et al*,[6] which narrowed down *TYK2* association to the three missense variants—P1104A, A928V and I684S—in RA and other autoimmune diseases through a fine-mapping strategy. The results are also consistent with the predictions of Polyphen-2 and SIFT tools, since common *TYK2* missense variant, V362F, was predicted to be benign while P1104A, A928V and I684S were damaging mutations.[30 31] In addition, the functional effect of P1104A and I684S variants (located in the kinase domains of the protein) has also been addressed by in vitro studies in primary T cells, B cells and fibroblasts. These studies showed that P1104A and I684S are catalytically impaired, leading to a reduced TYK2 activity and decreasing pro-inflammatory cytokines signalling, such as IL-6 or IL-12.[32 33] Nevertheless, since the three *TYK2* rare and low-frequency variants included in the present study were selected according to the detailed fine-mapping study performed by Diogo *et al* in a large RA study cohort, the genetic effect of additional independent rare and low-frequency *TYK2* variants cannot be ruled out in SSc susceptibility.

Interestingly, several IL-12 pathway-related genes have been reported to be associated with SSc: *IL12RB1* and *IL12RB2* (both IL-12-receptor chains), *IL12A* (p35 subunit of IL-12) and *STAT4* (the transcription factor of the IL-12 signalling axis).[3 4 8 9] Thus, the association of *TYK2* with SSc reported in the present study adds another piece of evidence showing the crucial role of this IL pathway in SSc pathogenesis.

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IL-12 is a pro-inflammatory cytokine that induces type 1 helper T cells (Th1) and, in combination with interferon (IFN)-γ, antagonises type 2 helper T cells (Th2) differentiation.[34] Serum levels of IL-12 are significantly increased in patients with SSc, and this overproduction has been associated with renal vascular damage.[35] In addition, functional studies have suggested that Th1 responses may be crucial in mediating early inflammatory processes in SSc. As stated above, P1104A, A928V and I684S missense variants are damaging *TYK2* mutations that ultimately lead to an impaired IL-12 signalling. This effect would be consistent with the protective effect observed for these variants and a lower SSc susceptibility. Thus, target therapies blocking this pathway could be an effective treatment for the disease, such as ustekinumab, an anti-IL-12/23 p40 monoclonal antibody currently approved for the treatment of psoriatic arthritis.[36–38]

Remarkably, pharmaceutical companies are setting their sight on JAK family as therapeutic targets for the treatment of autoimmune diseases, such as RA and type 1 diabetes, given its central role in the signalling pathways of a wide range of cytokines. Drug discovery research is focused on the development of specific JAK protein inhibitors, such as the recently approved JAK3 inhibitor, tofacitinib, for the treatment of RA.[39] TYK2 inhibitors have also been described, although none of these drugs have yet made it to the clinical trials.[40]

In summary, the present study identified *TYK2* as a novel susceptibility factor for SSc. Our results, together with previous findings, reinforce the crucial involvement of IL-12 signalling axis in the disease development; thus, this pathway might represent an attractive therapeutic target for the treatment of SSc.

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