

Polymorphisms in *TBX21* and *STAT4* Increase the Risk of Systemic Sclerosis

Evidence of Possible Gene–Gene Interaction and Alterations in Th1/Th2 Cytokines

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Objective. Systemic sclerosis (SSc) is an autoimmune disease characterized by fibrosis of the skin and internal organs. Dysregulation of the immune system, including the Th1/Th2 cytokine balance, is central to the pathogenesis of SSc. This study was undertaken to investigate the hypothesis that single-nucleotide polymorphisms (SNPs) in *TBX21* and *STAT4*, both of which are critical transcription factors that regulate the Th1/Th2 balance, are associated with SSc susceptibility.

Methods. We tested SNPs in *TBX21* and *STAT4* for association with SSc in 2 independent cohorts, the SSc Registry cohort (880 SSc cases and 507 controls)

and the University of Texas SSc cohort (522 cases and 531 controls). Additional white control genotypes were obtained from public repositories. We also investigated for gene–gene interactions. Plasma cytokines and whole blood gene expression profiles were examined to determine functional effects of these SNPs.

Results. Multiple SNPs in *TBX21* and *STAT4* were found to be associated with SSc. In a combined analysis of 902 SSc patients and 4,745 controls, TT genotyping of the *TBX21* rs11650354 variant revealed a recessive pattern for disease susceptibility ($P_{\text{corr}} = 1.4 \times 10^{-15}$, odds ratio 3.37, 95% confidence interval 2.4–4.6). In an analysis of 1,039 SSc patients and 3,322 controls, the A allele of the *STAT4* variant rs11889341 was associated with increased SSc susceptibility in a dominant pattern ($P_{\text{corr}} = 2.4 \times 10^{-5}$, odds ratio 1.29, 95% confidence interval 1.2–1.5). Furthermore, we identified gene–gene interaction among the *TBX21* and *STAT4* variants, such that the *STAT4* genotype increased the risk of SSc only in the *TBX21* CC genotype group. SSc patients carrying the *TBX21* CC genotype had higher interleukin-6 (IL-6) and tumor necrosis factor α levels, and those with the TT genotype had elevated IL-2, IL-5, IL-4, and IL-13 (Th2) levels, compared with controls. Whole blood expression profiles revealed dysregulation of type I interferon pathways in the CC group and T cell pathways in the TT group of the *TBX21* SNP.

Conclusion. The present results, from studies of 2 independent cohorts, indicate that SNPs in *TBX21* and *STAT4* contribute uniquely and interactively to SSc susceptibility, leading to altered cytokine balance and immune dysregulation.

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Systemic sclerosis (SSc; scleroderma) is a chronic multisystem disease of unknown etiology, which is clinically characterized by progressive fibrosis of the skin and internal organs, widespread small vessel obliteration, and autoimmunity. Although SSc is relatively uncommon, affecting ~400,000 North Americans and Europeans, the lack of disease-modifying treatment results in significant morbidity and mortality to the individual as well as substantial economic cost (1).

Central to understanding the pathogenesis of SSc is defining the genes and pathways leading to autoimmunity and inflammation, vascular damage, and extracellular matrix production. Several genetic polymorphisms have been associated with scleroderma in multiple case-control studies and a few family studies (2–8). Some of these genetic variants are associated with susceptibility for development of scleroderma, while others act as disease modifiers.

There is substantial evidence indicating that dysregulation is a vital process in the pathogenesis of SSc, particularly early in the disease process. An indicator of immune dysregulation in SSc patients is the presence of disease-specific, mutually exclusive autoantibodies. These antibodies, most commonly anticentromere (ACA), anti-topoisomerase I (anti-topo I), and anti-RNA polymerase III (anti-RNAP III), identify relatively distinct clinical subgroups, (9–13). There have been conflicting reports regarding the role of T cells and Th1/Th2 cytokine balance in SSc (14). Some studies have provided evidence in support of the notion of Th1 activation in the peripheral blood with increased levels of interferon- γ (IFN γ) (15–17), while others indicate a preferential involvement of Th2 cells in SSc with increased levels of interleukin-4 (IL-4) and IL-13 (16,18,19). This variation could reflect the clinical diversity in SSc (e.g., SSc-associated autoantibody subsets). Another possibility could be the difference in the genetic backgrounds of patients, resulting in distinct alterations in immune balance.

The transcription factor T-bet (T-box expressed in T cells) (*TBX21*) is a key transcriptional activator of Th1 cell differentiation. T-bet plays an essential role in Th1/Th2 balance, where it is the master regulator of Th1 cell fate through promotion of Th1 cytokines and inhibition of Th2 cytokines (20,21). In a recent study, *TBX21* polymorphisms were shown to be associated with rheumatoid arthritis (RA) (22), and in previous studies they were associated with asthma (a Th2-mediated disease characterized by overproduction of Th2 cytokines [IL-4, IL-5, and IL-13]) (25,26) and type 1 diabetes mellitus (DM) (27). Finally, the cytokine balance in mice defi-

cient in T-bet is skewed toward Th2 cytokines, and *tbx2*-null mice have displayed increased sensitivity to bleomycin-induced dermal sclerosis (28,29).

STAT-4 is another critical transcription factor involved in regulation of the Th1/Th2 cytokine balance. It is expressed in activated peripheral blood monocytes, dendritic cells, and macrophages and is up-regulated by IL-12. Upon binding of IL-12 to the IL-12 receptor, STAT-4 is phosphorylated and forms a homodimer that translocates to the nucleus, where it enhances production of Th1 cytokines such as IFN γ and suppresses production of Th2 cytokines such as IL-4, IL-5, and IL-13. Interestingly, *STAT4* also has been shown to be activated in response to type I IFNs, a cytokine network that is dysregulated in SSc (30). *STAT4* polymorphisms have been found to be associated with SSc (31) and other autoimmune diseases, including RA (32), systemic lupus erythematosus (SLE) (32), asthma (33), type 1 DM (34), and Sjögren's syndrome (SS) (35).

Given the potential importance of Th1/Th2 cytokine balance in SSc, we investigated the association of polymorphisms in the *TBX21* and *STAT4* genes with SSc. We demonstrated a significant association of both *TBX21* and *STAT4* polymorphisms with susceptibility to SSc in 2 large and independent cohorts. Further, we demonstrated gene-gene interaction between *TBX21* and *STAT4* variants. Moreover, the functional data suggested a Th2 cytokine profile in the *TBX21* mutation group, a proinflammatory profile in the *TBX21* wild-type group, and a Th1 profile in the *TBX21* wild-type and *STAT4* mutation groups.

PATIENTS AND METHODS

SSc patients and controls. Two independent cohorts of SSc patients and control subjects were used in the current study. The first cohort (SSc Registry cohort) consisted of 880 SSc patients and 507 healthy controls from the Scleroderma Family Registry and DNA Repository, a nationwide registry established in 1994 (36). The second cohort (UT Division cohort) consisted of 522 SSc patients and 531 healthy controls, mainly from Texas, evaluated at the University of Texas Health Science Center at Houston Rheumatology Division between 1986 and the present (10,37). All SSc patients fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) preliminary criteria for disease classification (38) or had at least 3 of the 5 features of CREST syndrome (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasias). SSc patients were further classified based on diffuse or limited skin involvement (39) and by autoantibody status. All subjects provided written informed consent, and the study was approved by the Committee for the Protection of Human Subjects of the University of Texas Health Science Center at Houston.

In order to increase the number of control subjects to attain a >1:1 case:control ratio, we used genotyping data from previously published genome-wide association studies in RA and SLE (32,40). Published data reported by Remmers et al (32) on the *STAT4* rs11889341 polymorphism, collected for the New York Cancer Project from control subjects of self-reported European ancestry, (41) (n = 2,635), were used. Genotype data on the *TBX21* rs11650354 single-nucleotide polymorphism (SNP) were obtained from 3,172 control samples (all self-described North Americans of European descent) from studies 64–67 in the publicly available iControlDB database (www.illumina.com/pages.ilmn?ID=231). Additionally, data on *TBX21* rs11650354 SNP genotypes in 1,094 control subjects were obtained from the prostate cancer study (42) in the Cancer Genetic Markers of Susceptibility (CGEMS) project (<http://cgems.cancer.gov>).

To address the issue of population stratification between the 2 study cohorts, we compared the control frequencies in the SSc Registry cohort with control frequencies in the UT Division group, for all 13 *TBX21* and 5 *STAT4* polymorphisms. There were no statistically significant differences between the 2 control cohorts in any of the polymorphisms (see Supplementary Table A, available in the online version of this article at <http://www3.interscience.wiley.com/journal/76509746/home>).

Autoantibody analysis. All SSc patients were tested for antinuclear antibodies, using indirect immunofluorescence (Antibodies Inc., Davis, CA). ACAs were determined by their distinctive pattern on indirect immunofluorescence (43). Anti-topo I antibodies were determined by immunodiffusion (Inova Diagnostics, San Diego, CA). Anti-RNAP III antibodies were determined by enzyme-linked immunosorbent assay (ELISA; MBL, Nagoya, Japan).

SNP selection. SNPs were selected from the *TBX21* gene-transcribed sequence and 8,000 bp upstream and downstream regions. SNPs with minor allele frequencies of >5% in the Centre d'Etude du Polymorphisme Humain from Utah population sample (Utah residents with ancestry from northern and western Europe) and a coefficient of determination of $r^2 = 0.8$ were identified based on data from HapMap (44) (rs9910408, rs11079786, rs4794067, rs10514934, rs16946264, rs11650354, rs11657479, rs7502875, rs16947058, rs16947078, and rs17699436). Two additional coding region SNPs (rs2240017 and rs2074190) were also selected. SNPs on the *STAT4* gene were selected based on the most significant *STAT4* variants identified in the candidate gene study in RA and SLE (32) (rs11889341, rs7574865, rs8179673, rs10181656, and rs6752770).

SNP genotyping. Genomic DNA was extracted from peripheral blood with the PureGene genomic DNA isolation kit (Gentra Systems, Minneapolis, MN). The SNPs were genotyped with an ABI TaqMan SNP genotyping assay using a ABI 7900HT real-time thermocycler (Applied Biosystems, Foster City, CA). Automated allele calling was performed with allelic discrimination plots using SDS 2.3 software from Applied Biosystems. Multiple positive Centre d'Etude du Polymorphisme Humain DNA samples from Coriell Institute for Medical Research (Camden, NJ) and negative controls were used in each genotyping assay, and allele calls were verified with HapMap data for validation. Confirmation of the TaqMan genotyping was performed by bidirectional direct se-

quencing using 32 samples (24 SSc patient and 8 control samples). Visual inspection of sequences confirmed 100% concordance with results obtained by TaqMan assay (for detailed description of methods, see supplementary material, available in the online version of this article at <http://www3.interscience.wiley.com/journal/76509746/home>).

ELISA for cytokines. Plasma was collected in EDTA and stored at -80°C for bulk analysis using electrochemiluminescence multiplex assays (Meso Scale Discovery, Gaithersburg, MD) to determine levels of IFN γ , tumor necrosis factor α (TNF α), IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17, and IL-23 (45). Sample cytokine concentrations were determined with MSD Workbench 3.0 software.

Gene expression array of peripheral white blood cells and data analysis. Blood samples were drawn directly into PAXgene tubes (PreAnalytiX, Franklin Lakes, NJ), and total RNA was isolated. The amplified chromosomal RNA was hybridized on Human Ref-8 v2 arrays (Illumina, San Diego, CA), and the data were extracted with the Beadstudio software suite (Illumina). Raw data were also analyzed in BRB Array-Tools, version 3.7 (National Cancer Institute), developed by Dr. Richard Simon and Amy Peng Lam and the BRB Array-Tools Development Team (see detailed methods in supplementary material, <http://www3.interscience.wiley.com/journal/76509746/home>).

Statistical analysis. Statistical analyses were performed using SAS 9.1.3 (SAS Institute, Cary, NC). Allelic and genotypic associations were calculated using the standard Pearson's chi-square test or, when appropriate, Fisher's exact test. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were calculated. The Mantel-Haenszel chi-square test was used to combine data from the 2 independent cohorts. *P* values less than 0.05 were considered significant. To account for multiple comparisons, Bonferroni correction was applied during the model-building phase. Logistic regression analysis was performed to identify significant independent risk factors for SSc. Measures of pairwise linkage disequilibrium (LD) were determined using Haploview (Whitehead Institute, Cambridge, MA). Classification and Regression Tree (CART) analysis (46) was performed to explore gene-gene interactions (CART 6.0; Salford Systems, San Diego, CA). Race, sex, and variants of *TBX21* and *STAT4* genes were used as nominal categorical variables to predict the outcome SSc. The cytokine values were compared using the Wilcoxon Mann-Whitney U test (detailed methods in supplementary material, <http://www3.interscience.wiley.com/journal/76509746/home>).

RESULTS

We conducted this candidate gene study using the SSc Registry cohort (36) and replicated it in an independent cohort (UT Division) (10,37). Clinical and serologic characteristics of the SSc patients from both of these cohorts are presented in Table 1. All *TBX21* gene SNPs studied were in Hardy-Weinberg equilibrium in white controls. SNPs rs10514934 and rs16947078 in black controls and SNP rs17699436 in Hispanic controls were not in Hardy-Weinberg equilibrium and were

Table 1. Characteristics of the control subjects and the patients with systemic sclerosis (SSc)

	SSc Registry cohort			UT Division cohort		
	White	Black	Hispanic	White	Black	Hispanic
Controls						
Sex						
n*	421	63	23	277	138	116
Female, no. (%)	198 (47.0)	36 (57.1)	15 (65.2)	155 (56.6)	106 (77.4)	65 (56.0)
Male, no. (%)	223 (53.0)	27 (42.9)	8 (34.8)	119 (43.4)	31 (22.6)	51 (44.0)
SSc patients						
Sex						
n	745	69	66	314	108	100
Female, no. (%)	676 (90.7)	61 (88.4)	58 (87.9)	259 (82.5)	90 (83.3)	90 (90.0)
Male, no. (%)	69 (9.3)	8 (11.6)	8 (12.1)	55 (17.5)	18 (16.7)	10 (10.0)
Skin involvement						
n	712	67	60	292	97	97
Limited cutaneous SSc, no. (%)	465 (65.3)	25 (37.3)	28 (46.7)	146 (50.0)	28 (28.9)	38 (39.2)
Diffuse cutaneous SSc, no. (%)	247 (34.7)	42 (62.7)	32 (53.3)	146 (50.0)	69 (71.1)	59 (60.8)
Antibodies						
n	509	43	45	158	43	60
Anticentromere, no. (%)	221 (43.4)	4 (9.3)	12 (26.7)	79 (50.0)	8 (18.6)	17 (28.3)
Anti-topoisomerase I, no. (%)	138 (27.1)	22 (51.2)	14 (31.1)	36 (22.8)	21 (48.8)	25 (41.7)
Anti-RNA polymerase III, no. (%)	150 (29.5)	17 (39.5)	19 (42.2)	43 (27.2)	14 (32.6)	18 (30.0)

* Data missing in 3 white controls and 1 black control in the University of Texas SSc cohort (UT Division cohort).

removed from the analysis in these ethnic groups. All studied SNPs in the *STAT4* gene were in Hardy-Weinberg equilibrium in all the 3 ethnic groups (see Supplementary Table 1, <http://www3.interscience.wiley.com/journal/76509746/home>).

LD and haplotype block structure in *TBX21* and *STAT4* genes. Pairwise LD was calculated by both D' and r^2 for the 13 *TBX21* SNPs and 5 *STAT4* SNPs typed in white, black, and Hispanic controls (see Supplementary Figure 1, <http://www3.interscience.wiley.com/journal/76509746/home>). LD in whites ($r^2 > 0.8$) was identified for the following pairs of *TBX21* SNPs: rs11079786 and rs4794067, rs11079786 and rs2074190, rs4794067 and rs2074190, rs11657479 and rs7502875, rs11657479 and rs16947078, and rs7502875 and rs16947078. The 4 *STAT4* SNPs rs11889341, rs7574865, rs8179673, and rs10181656 demonstrated high LD with one another in all 3 ethnic groups ($r^2 > 0.95$).

***TBX21* and *STAT4* SNP association analysis.** Two of the 13 variants in the *TBX21* gene, rs11650354 and rs17699436, demonstrated significant association with SSc in whites in both the SSc Registry and UT Division cohorts. These 2 variants exhibited association at the genotypic and allelic levels, respectively. When the 2 cohorts were combined using Mantel-Haenszel analysis, these 2 variations in *TBX21* still exhibited strong association with SSc in whites, with rs11650354 being a susceptibility factor and rs17699436 playing a protective role (Table 2). Thus, we were able to demonstrate and

replicate the association of the *TBX21* gene variants rs11650354 and rs17699436 in the 2 independent cohorts.

All of the *STAT4* variants exhibited increased minor allele frequencies in white SSc subjects in both cohorts. When the 2 cohorts were combined using Mantel-Haenszel analysis, all 5 variants remained significantly associated with SSc (Table 2).

The *TBX21* and *STAT4* SNP data in black and Hispanic subjects did not reach statistical significance. Data on these subjects are presented in Supplementary Tables 2 and 3.

***TBX21* rs11650354-rs17699436 diplotype association analysis.** Study of the haplotypes for the 2 replicating SNPs, rs11650354-rs17699436, revealed no significant difference in the CA (wild-type) haplotype frequency between SSc cases and controls. The CG haplotype was protective against SSc (7.0%; versus 10.7% in controls) whereas the TA haplotype was a susceptibility factor in SSc (19.0%; versus 15.4% in controls). Interestingly, the TG haplotype, carrying the mutation for both of the SNPs, was found to be a susceptibility factor in SSc, albeit in a very low frequency (0.6%; versus 0.01% in controls), suggesting that the 2 *TBX21* variants were acting independently of one another.

CART analysis for gene-gene interaction. An established exploratory method (CART) (47) was used to explore for gene-gene interactions in *TBX21* and *STAT4* genes in the 2 cohorts combined. All of the *TBX21* and *STAT4* polymorphisms were entered as

Table 2. Distribution of *TBX21* and *STAT4* SNP genotypes in North American white control subjects and patients with SSc*

SNP (minor allele)	Position, bp	SSc Registry cohort						UT Division cohort						Mantel-Haenszel-combined cohorts					
		MAF			P†	OR	P‡	MAF			P†	OR	P‡	OR			P†	OR	P‡
		Controls	SSc	OR				Controls	SSc	OR				(95% CI)†	(95% CI)‡				
<i>TBX21</i> §																			
rs9910408 (G)	43157873	0.44	0.40	0.83	0.14	0.06	0.40	0.45	1.22	0.01	0.10	0.96 (0.8–1.2)	0.65	0.97 (0.8–1.1)	0.66				
rs11079786 (T)	43160915	0.26	0.28	1.09	0.72	0.42	0.31	0.31	1.01	0.51	0.97	1.07 (0.9–1.3)	0.52	1.05 (0.9–1.2)	0.52				
rs4794067 (G)	43163827	0.26	0.29	1.13	0.54	0.29	0.29	0.30	1.04	0.61	0.77	1.11 (0.9–1.4)	0.33	1.09 (0.9–1.3)	0.32				
rs2240017 (G)	43165918	0.03	0.03	0.84	0.52	0.53	0.04	0.03	0.71	0.64	0.32	0.78 (0.5–1.2)	0.27	0.78 (0.5–1.2)	0.26				
rs2074190 (C)	43166209	0.25	0.28	1.16	0.47	0.26	0.30	0.30	1.01	0.73	0.97	1.10 (0.9–1.4)	0.41	1.08 (0.9–1.3)	0.41				
rs10514934 (G)	43167123	0.11	0.12	1.14	0.68	0.39	0.12	0.13	1.04	0.01	0.82	1.11 (0.9–1.4)	0.43	1.10 (0.9–1.4)	0.42				
rs16946264 (T)	43168433	0.10	0.10	0.91	0.75	0.54	0.13	0.08	0.64	0.01	0.02	0.78 (0.6–1.01)	0.06	0.79 (0.6–1.01)	0.06				
rs11650354 (T)	43177091	0.15	0.18	1.29	0.002	0.08	0.16	0.22	1.49	0.02	0.01	1.44 (1.1–1.8)	0.006	1.38 (1.1–1.7)	0.003				
rs11657479 (C)	43177900	0.22	0.25	1.24	0.12	0.06	0.25	0.29	1.22	0.35	0.13	1.29 (1.1–1.6)	0.016	1.23 (1.04–1.5)	0.015				
rs7502875 (C)	43178226	0.21	0.24	1.17	0.29	0.17	0.24	0.27	1.14	0.63	0.32	1.19 (0.97–1.5)	0.10	1.16 (0.98–1.4)	0.09				
rs16947058 (T)	43180185	0.42	0.42	0.99	0.99	0.94	0.46	0.45	0.94	0.93	0.71	0.97 (0.8–1.2)	0.78	0.97 (0.8–1.1)	0.71				
rs16947078 (G)	43180499	0.20	0.22	1.10	0.70	0.40	0.23	0.23	1.00	0.77	0.97	1.07 (0.9–1.3)	0.51	1.06 (0.9–1.3)	0.50				
rs17699436 (G)	43183574	0.11	0.08	0.72	0.10	0.04	0.10	0.06	0.55	0.02	0.01	0.64 (0.5–0.8)	0.002	0.66 (0.5–0.9)	0.001				
<i>STAT4</i> ¶																			
rs11889341 (A)	191651987	0.22	0.26	1.30	0.03	0.01	0.24	0.28	1.22	0.36	0.14	1.33 (1.1–1.6)	0.005	1.27 (1.1–1.5)	0.004				
rs7574865 (A)	191672878	0.21	0.26	1.31	0.03	0.01	0.25	0.28	1.18	0.48	0.21	1.34 (1.1–1.6)	0.005	1.26 (1.1–1.5)	0.004				
rs8179673 (C)	191677586	0.22	0.27	1.36	0.01	0.003	0.25	0.29	1.20	0.39	0.16	1.37 (1.1–1.7)	0.002	1.30 (1.1–1.5)	0.001				
rs10181656 (C)	191678124	0.22	0.27	1.33	0.02	0.01	0.25	0.29	1.21	0.37	0.15	1.37 (1.1–1.6)	0.002	1.29 (1.1–1.5)	0.002				
rs6752770 (C)	191681808	0.29	0.30	1.05	0.49	0.59	0.28	0.37	1.50	0.004	0.001	1.25 (1.1–1.5)	0.017	1.20 (1.03–1.4)	0.02				

* Odds ratios (ORs) and 95% confidence intervals (95% CIs) are for carriage of the minor allele genotype. Control subjects were used as reference for all comparisons. SNP = single-nucleotide polymorphism; SSc = systemic sclerosis; MAF = minor allele frequency; UT Division cohort = University of Texas SSc cohort.

† Genotypic 3 × 2 chi-square comparison.

‡ Allelic comparison.

§ Chromosome 17.

¶ Chromosome 2.

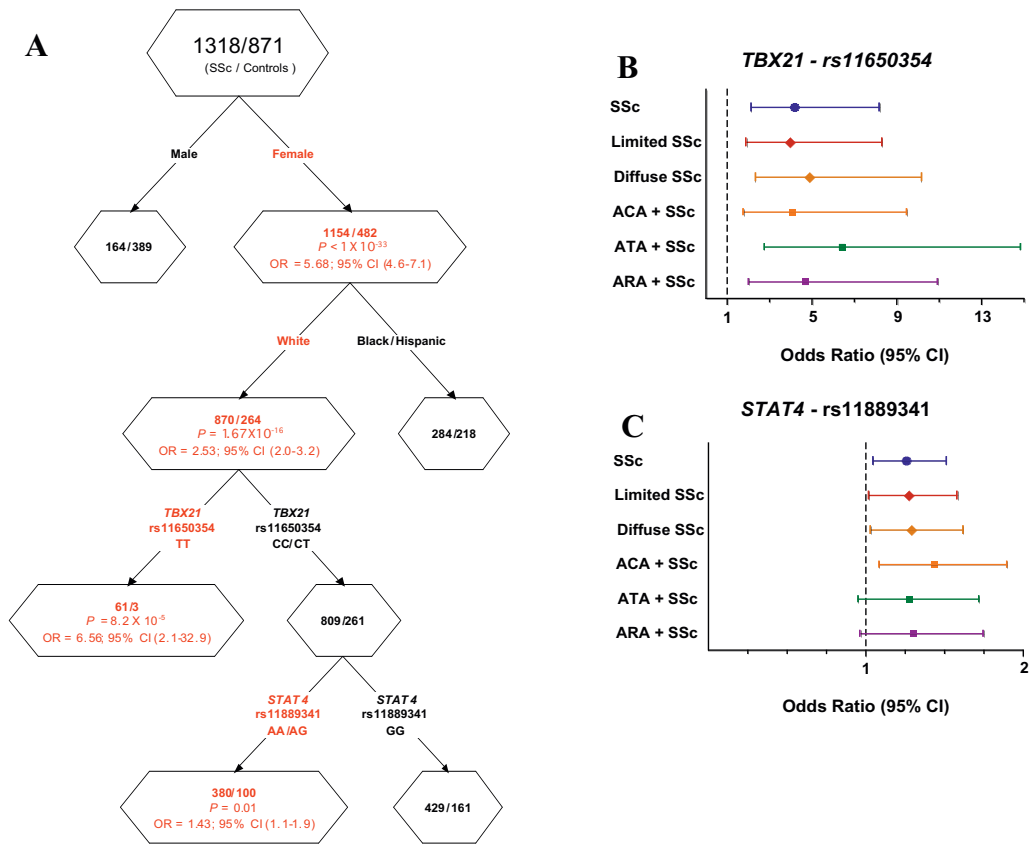


Figure 1. A, Cartesian and Regression Tree analysis showing a gene-gene interaction between *TBX21* and *STAT4* polymorphisms in systemic sclerosis (SSc). Red text denotes SSc susceptibility factors. B and C, Estimated risk of *TBX21* single-nucleotide polymorphism (SNP) rs11650354 (B) and *STAT4* SNP rs11889341 (C) in SSc patients, by logistic regression analysis. The analysis was controlled for the confounding effects of sex and race. Control subjects were used as reference for all comparisons. The *TBX21* SNP showed a recessive mode of inheritance, and the *STAT4* SNP showed a dominant mode of inheritance. OR = odds ratio; 95% CI = 95% confidence interval; ACA = anticentromere antibody; ATA = anti-topoisomerase I antibody; ARA = anti-RNA polymerase III antibody.

variables in the CART model, along with race and sex. CART analysis interactively segregates subjects into 2 subgroups using the most powerful variable classifier for SSc. The variables partitioning out higher up in the decision tree suggest greater importance than the ones lower in the tree.

Figure 1A depicts female sex and white race as the first 2 splits. In white female subjects, the rs11650354 TT variant increased risk for SSc (OR 6.56 [95% CI 2.1–32.9]) consistent with a recessive effect. Individuals with the CC/CT genotype were further split into 2 subgroups based on the *STAT4* variant (rs11889341) genotype. The AA/AG genotype of the *STAT4* variant increased risk for SSc as compared with the GG wild-type genotype (OR 1.43 [95% CI 1.1–1.9]). Therefore, this analysis suggested a more prominent role of the

TBX21 rs11650354 SNP and also revealed a potential gene-gene interaction between *TBX21* and *STAT4* genes among white female patients with SSc.

Logistic regression analysis in white subjects after controlling for sex demonstrated significant *P* values for *TBX21* CC/CT-*STAT4* AA/AG (OR 1.36 [95% CI 1.1–1.7]) and *TBX21* TT-*STAT4* GG (OR 5.7 [95% CI 2.2–15.0]) as compared with the *TBX21* CC/CT-*STAT4* GG group. Results in the *TBX21* TT-*STAT4* AA/AG group were not statistically significant. Thus, the logistic regression analysis confirmed the gene-gene interaction observed in the CART analysis.

***TBX21* and *STAT4* polymorphisms in the SSc Registry and UT Division cohorts and additional controls.** *TBX21* SNP rs11650354 and *STAT4* SNP rs11889341 were selected for further analysis based on

Table 3. Best fit model for distribution of the *TBX21* SNP rs11650354 genotype in North American white control subjects and patients with SSc*

	SSc Registry cohort				UT Division cohort				Combined cohorts				SSc Registry and UT Division cohorts + iControlDB and CGEMS controls†								
	n	%	TT, CC/CT, %	P	OR (95% CI)	n	%	TT, CC/CT, %	P	OR (95% CI)	n	%	TT, CC/CT, %	P	OR (95% CI)	n	%	TT, CC/CT, %	P	OR (95% CI)	
Control	252	2.4	97.6			227	1.3	98.7			479					4,745					
SSc patients	596	8.6	91.4	0.003	3.84 (1.6–10.1)	306	5.6	94.4	0.03	4.39 (1.2–19.1)	902	3.9	92.2	3.9×10^{-5}	4.26 (2.0–9.2)	902	1.4	91.4	1.4×10^{-15}	3.37 (2.4–4.6)	
Limited cutaneous SSc	364	9.1	90.9	0.002	4.09 (1.6–11.0)	147	4.8	95.2	0.12	3.73 (0.8–18.5)	511	4.8	92.2	4.8×10^{-5}	4.44 (2.0–9.9)	511	7.2	90.9	7.2×10^{-12}	3.51 (2.4–5.1)	
Diffuse cutaneous SSc	189	9	91	0.006	4.05 (1.5–11.8)	138	7.2	92.8	0.009	5.83 (1.4–27.2)	327	5.1	92.8	5.1×10^{-5}	4.70 (2.1–10.9)	327	8.1	90.9	8.1×10^{-10}	3.70 (2.3–5.8)	
Antibodies																					
Anticentromere	178	10.7	89.3	0.0009	4.92 (1.8–14.0)	88	4.5	95.5	0.24	3.56 (0.7–20.4)	266	3.9	95.5	3.9×10^{-5}	4.94 (2.1–11.7)	266	2.2	95.5	2.2×10^{-9}	3.92 (2.4–6.3)	
Anti-topoisomerase I	102	7.8	92.2	0.05	3.49 (1.1–11.7)	34	5.9	94.1	0.21	4.67 (0.5–36.2)	136	0.003	94.1	0.003	4.14 (1.5–11.4)	136	0.0006	0.0006	3.27 (1.6–6.6)		
Anti-RNA polymerase III	132	10.6	89.4	0.001	4.86 (1.7–14.6)	34	5.9	94.1	0.21	4.67 (0.5–36.2)	166	2.4	94.1	2.4×10^{-4}	5.57 (2.2–14.0)	166	2.2	94.1	2.2×10^{-8}	4.41 (2.4–7.8)	

* ORs and 95% CIs are for carriage of the TT genotype. Control subjects were used as reference for all comparisons. P values were corrected for multiple model testing using the Bonferroni adjustment. See Table 2 for other definitions.

† The Illumina iControlDB database was queried to ascertain the SNP genotype frequencies in 3,172 white controls (CC 2,232 [70.4%], CT 863 [27.2%], TT 77 [2.4%]). The Cancer Genetic Markers of Susceptibility (CGEMS) database was queried to ascertain the SNP genotype frequencies in 1,094 white controls (CC 752 [68.7%], CT 316 [28.9%], TT 26 [2.4%]).

their association with SSc as determined by CART, logistic regression, and chi-square analyses. As seen in Table 3, the *TBX21* SNP rs11650354 depicted a recessive model and a significant association in both cohorts individually and combined.

To further increase the ratio of cases to controls, additional genotypes from North American controls of European ancestry were obtained from public repositories (40,42). The TT genotype frequency in controls was similar between our combined cohort (1.9%) and the publicly available data on controls (iControlDB 2.4%, CGEMS 2.4%; $P = 0.75$ and $P = 0.58$, respectively), thus demonstrating that the controls in the publicly available databases were similar to our control cohorts and these could be combined for joint analyses. In a comparison of the genotype frequencies in 4,745 controls and 902 SSc cases, a highly significant association was observed (corrected $P [P_{\text{corr}}] = 1.4 \times 10^{-15}$, OR 3.37, 95% CI 2.4–4.6) (Table 3). Logistic regression analysis revealed a recessive model as the best fit and confirmed that the *TBX21* TT genotype was an independent risk factor for SSc and its subsets after controlling for the confounding effects of sex and race (Figure 1B).

Similarly, the frequency of the *STAT4* variant rs11889341 in controls was obtained from published data on North Americans of European ancestry (32). The A allele frequency in controls was similar between our combined cohort (22.7%) and the published frequency (22%) ($P = 0.57$), thus suggesting that the controls in the publicly available databases were similar to our control cohorts and the two could be combined for joint analyses. On comparing the allelic frequencies in 3,322 controls and 1,039 SSc cases, a significant association was observed ($P_{\text{corr}} = 2.4 \times 10^{-5}$, OR 1.29, 95% CI 1.2–1.5) (Table 4). Logistic regression analysis revealed a dominant model as the best fit and confirmed that the *STAT4* AA/AG genotype was an independent risk factor for SSc and its subsets after controlling for the confounding effects of sex and race (Figure 1C).

The associations of the *TBX21* SNP rs11650354 and the *STAT4* SNP rs11889341 were observed for SSc overall, and not for any specific clinical or autoantibody subset. We observed an association of the *TBX21* rs11650354 variant with SSc both with and without pulmonary fibrosis, as compared with controls. There was no significant association between the TT genotype and the presence of pulmonary fibrosis when compared with patients without pulmonary fibrosis. These *TBX21* and *STAT4* SNPs were tested in black and Hispanic populations separately, and the results did not reach

statistical significance in skin and autoantibody subset analyses (see Supplementary Tables 5 and 6, available in the online version of this article at <http://www3.interscience.wiley.com/journal/76509746/home>).

Association of the *TBX21* variant rs11650354 with plasma cytokine levels. To better understand the immunologic significance of the *TBX21* variant rs11650354, plasma levels of 13 cytokines were determined. SSc patient ($n = 273$) and control ($n = 123$) samples were stratified based on their genotypes (CC group and TT group). Compared with controls in the CC group, SSc patients in the CC group had higher levels of circulating IL-6 and TNF α (Figure 2A); however, no differences in levels of IL-4, IL-5, IL-13, or IL-2 were observed. In contrast, in the TT group, SSc patients had significantly elevated levels of circulating IL-2 and IL-5 and a trend toward an increase in IL-4 and IL-13 levels compared with controls (Figure 2B), with no increase in levels of IL-6 or TNF α . The remaining cytokines did not show a trend toward association in either of the groups. These findings suggest that patients in the *TBX21* mutation group (TT) have a more prominent Th2 cytokine profile, while patients in the wild-type group (CC) have a more prominent proinflammatory cytokine profile.

Whole blood RNA gene expression profiling. Next, we compared whole blood gene expression profiles of SSc patients and healthy controls stratified into CC (3 SSc patients and 3 controls) and TT (3 SSc and 2 controls) groups based on *TBX21* genotype. We examined known biologic pathways to increase the study's power to detect dysregulated gene networks. Of a total of 258 biologic pathways from the BioCarta database, 12 and 22 pathways in the CC and TT groups, respectively, were found to be differentially regulated at $P < 0.05$. The top 5 differentially regulated pathways in SSc are shown in Figures 2C and D. The most significantly differentially regulated pathway in the CC group was the IFN α signaling pathway. In the TT group, both T cell and B cell pathways were among those most significantly differentially regulated. These data support our observations in the studies of plasma cytokine networks and provide evidence suggesting a role of type I IFN and proinflammatory pathways in the CC group and T cell pathways in the TT group.

Association of the *STAT4* variant rs11889341 with plasma cytokine levels. We observed a gene–gene interaction between the *STAT4* variant rs11889341 and the CC/CT genotype of the *TBX21* variant rs11650354, by CART analysis (Figure 1A). In the *TBX21* wild-type

Table 4. Best fit model for distribution of the *STAT4* SNP rs11889341 alleles in North American white control subjects and patients with SSC*

	SSc Registry cohort				UT Division cohort				SSc Registry + UT Division + control†			
	2n	A, %	P	OR (95% CI)	2n	A, %	P	OR (95% CI)	2n	A, %	P	OR (95% CI)
Control subjects	826	21.7			548	24.3			6,644	22.2		
SSc patients	1,466	26.4	0.03‡	1.30 (1.1-1.6)	612	28.1	0.14	1.22 (0.9-1.6)	2,078	26.9	2.4×10^{-5} ‡	1.29 (1.2-1.5)
Limited cutaneous SSC	914	25.5	0.06	1.24 (0.99-1.5)	284	30.6	0.15‡	1.38 (1.0-1.9)	1,198	26.7	1.7×10^{-3} ‡	1.28 (1.1-1.5)
Diffuse cutaneous SSC	488	28.1	0.03‡	1.41 (1.1-1.8)	284	26.8	0.43	1.14 (0.8-1.6)	772	27.6	2.0×10^{-3} ‡	1.34 (1.1-1.6)
Antibodies												
Anticentromere	430	26.3	0.07	1.29 (0.98-1.7)	154	31.2	0.08	1.41 (0.95-2.1)	584	27.6	0.009‡	1.34 (1.1-1.6)
Anti-topoisomerase I	272	26.5	0.10	1.30 (0.9-1.8)	72	23.6	0.90	1.04 (0.6-1.8)	344	25.9	0.10‡	1.23 (0.95-1.6)
Anti-RNA polymerase III	298	26.8	0.07	1.33 (0.98-1.8)	82	26.8	0.62	1.14 (0.7-1.9)	380	26.8	0.09‡	1.29 (1.01-1.8)

* ORs and 95% CIs are for carriage of the A allele. Control subjects were used as reference for all comparisons. See Table 2 for definitions.

† Published frequencies in controls were ascertained for 2,635 white control subjects from the New York Cancer Project.

‡ Corrected for multiple model testing using the Bonferroni adjustment.

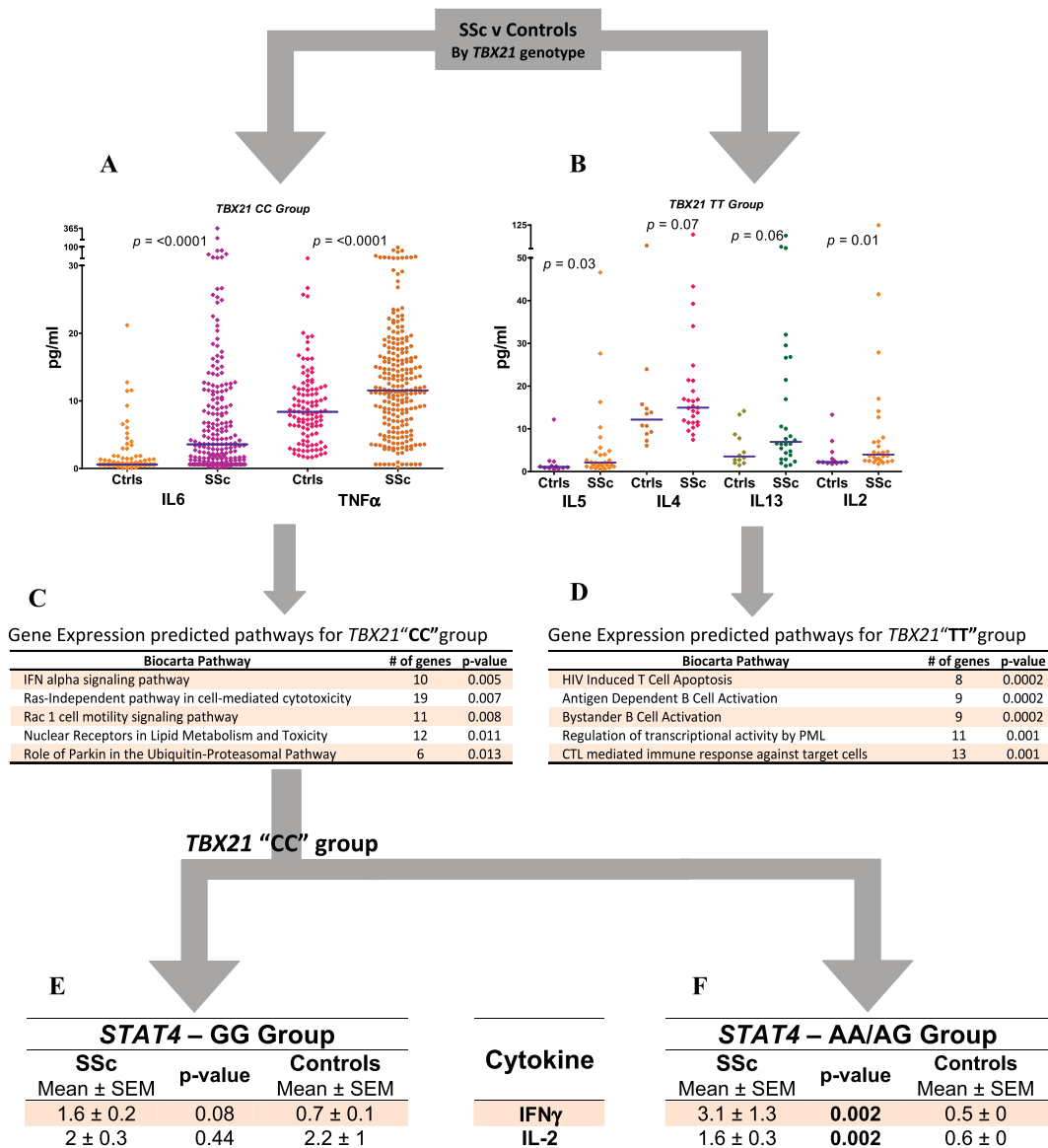


Figure 2. A–D, Plasma cytokine levels (A and B) and gene expression predicted pathways (C and D) by *TBX21* single-nucleotide polymorphism (SNP) group in systemic sclerosis (SSc) patients compared with healthy controls (Ctrls). Bars in A and B show the means. *P* values for gene expression predicted pathways were determined by Fisher’s least significant statistic after 100,000 permutations (<http://cgap.nci.nih.gov/Pathways/BioCarta/>). E and F, Gene expression predicted pathways by *STAT4* SNP group in SSc patients compared with healthy controls. IL-6 = interleukin-6; TNF α = tumor necrosis factor α ; IFN = interferon; HIV = human immunodeficiency virus; PML = promyelocytic leukemia; CTL = cytotoxic T lymphocyte.

(CC) subset, plasma IFN γ and IL-2 levels were increased in the AA/AG group of SSc patients as compared with healthy controls. Levels of these cytokines were not significantly different between SSc patients and controls in the GG group. TNF α and IL-6 were increased in SSc patients compared with controls in both

of the *STAT4* groups. The rest of the cytokines did not show a trend toward association in either of the groups. These data suggest that in SSc patients with the *TBX21* wild-type genotype, the *STAT4* variation is associated with alterations in circulating T cell cytokines, leading to a Th1 cytokine profile.

DISCUSSION

The present data, obtained using 2 independent cohorts, clearly demonstrate an association of *TBX21* and *STAT4* polymorphisms with SSc. We also showed a gene–gene interaction among the *TBX21* and *STAT4* variants, such that the *STAT4* genotype increased the risk for SSc only in the group with the *TBX21* wild-type genotype. Furthermore, we demonstrated distinct cytokine profiles based on the *TBX21* and *STAT4* genotypes suggestive of genetic influence on the complex immune balance and dysregulation of distinct gene expression pathways in each group.

Findings of recent genetic studies support the emerging concept that distinct clinical autoimmune diseases may share genetic susceptibility factors. Consistent with this, some of the genes implicated in SSc are also associated with susceptibility to other autoimmune diseases such as RA and SLE, including the HLA class II gene family (9,37,48), protein tyrosine phosphatase N22, and *STAT4* (2). Associations of *STAT4* polymorphisms with SSc (31), RA (32), SLE (32), type 1 DM (34), SS (35), and asthma (33), all of which are autoimmune disorders, have been reported. In the current study, we confirmed an association of *STAT4* mutations with susceptibility to SSc. These data suggest that *STAT4* may be an “autoimmune disease susceptibility gene” and support the concept of common dysregulated pathways across multiple autoimmune diseases. In contrast, other genes, such as those for allograft inflammatory factor 1 (3) and fibrillin 1 (8), have been reported in association with SSc only, with no reports to date of associations with RA and SLE.

The present report is the first to describe an association of *TBX21* variants (rs11650354 and rs17699436) with SSc in North American whites. *TBX21* polymorphisms have been recently reported to be associated with susceptibility to RA (22) and have also been found to be associated with asthma (25,26) and DM (27), although associations with SLE or SS have not been reported to date. These findings indicate that *TBX21* might possibly be placed in the category of “autoimmune disease susceptibility gene,” and its potential role in other autoimmune diseases needs to be investigated.

An important feature of our study was the use of CART analysis to identify significant gene–gene interactions. CART analysis revealed a major gene–gene interaction between the *TBX21* CC/CT genotype and the *STAT4* rs11889341 variant, which was further confirmed with logistic regression analysis. The interaction

between *STAT4* and *TBX21* is intriguing given the fact that both of these genes are involved in IL-12 signaling and regulation of the Th1/Th2 cytokine balance. Upon IL-12 stimulation of its receptor, STAT-4 forms an active homodimer that translocates to the nucleus to enhance *TBX21* transcription, which subsequently serves as a major regulator of the Th1 pathway. We observed that a *STAT4* mutation was important only in patients without the *TBX21* mutation. This suggests that there may be independent perturbations at multiple points in the IL-12 pathway that contribute to SSc susceptibility. Thus, genetic alterations in *TBX21* could lead to skewing of the Th1/Th2 cytokine balance toward Th2 cytokines. In the absence of *TBX21* mutation, genetic alterations of *STAT4*, an upstream gene in the same pathway, could exert an effect leading to a Th1 cytokine profile.

Alterations in the Th1/Th2 cytokine balance have been demonstrated in some patients with SSc. These findings in humans are supported by the findings of 2 studies using the bleomycin-induced skin fibrosis model, which demonstrated the development of more severe disease in T-bet–deficient (Th2-prone) mice (28,49). Recent observations have suggested that in a subset of SSc patients, there is an increase in type I IFN pathways, similar to that observed in SLE (30). We hypothesize that in the presence of the *TBX21* polymorphism, CD4+ T cells fail to differentiate into the Th1 lineage and default to a Th2 fate, leading to a Th2-predominant environment as suggested by the increase in levels of IL-4, IL-5, and IL-13 in plasma, while the *STAT4* polymorphism group directs the CD4+ T cells toward a Th1 lineage, as highlighted by the increase in IL-2 and IFN γ levels. In contrast, the *TBX21* CC variant is associated with alterations in type I IFNs, IL-6, and TNF α , suggesting a potential role of innate immune cells and proinflammatory pathways in this group of SSc patients. Our findings linking the *TBX21* polymorphism with a Th2 cytokine profile in SSc are not only relevant to SSc, but could extend to other fibrotic and inflammatory diseases, e.g., asthma.

The potential influence of *TBX21* polymorphisms in the Th1/Th2 cytokine balance observed in our study may also be of importance in asthma, a disease in which Th2 cytokines play an important role. Indeed, several studies have shown association of *TBX21* polymorphisms with susceptibility to asthma and response to corticosteroids in children with asthma (50). It will be of interest to determine if similar alterations in Th1/Th2 cytokines are observed in asthma patients based on the polymorphisms in *TBX21* as were observed in SSc patients in the current study. Interestingly, peripheral

blood levels of T-bet messenger RNA (mRNA) have been reported to be lower in asthma patients than in controls (51). In the current study, we did not detect the *TBX21* transcripts in the whole blood gene expression array in any of the samples, perhaps due to low expression levels. Further studies need to be undertaken to measure T-bet mRNA in SSc patients. If the mRNA levels are found to be dysregulated in SSc, this knowledge could be invaluable in developing therapies for a fibrotic disease driven by inflammation such as scleroderma (28,52).

The current study was limited by relatively small numbers of black and Hispanic subjects as compared with whites, who were from 2 large, independent, and well-established case-control cohorts. The SNP frequencies in blacks and Hispanics trended in the same direction as those in whites, and studies of larger cohorts of SSc patients and controls of these ethnic backgrounds will be necessary to carry these results further. The data-driven observation of a gene-gene interaction by CART analysis is an exploratory finding and is hypothesis generating, requiring confirmation in a much larger study. Another limitation is the lack of complete information regarding the causal polymorphisms and their exact functional roles. The SNPs in *TBX21* and *STAT4* are located in introns, which could be a site for potential splice variation and/or binding of regulatory elements. Overall, our results affirm the use of replicative cohorts along with multiple statistical approaches rather than a single methodology, as the optimal strategy to elucidate complex gene interactions in polygenic diseases.

In summary, the current study clearly demonstrates that *TBX21* and *STAT4* contributed uniquely and interactively to susceptibility to the development of SSc in 2 independent cohorts. Our data provide evidence that there is a unique subset of SSc patients defined by specific gene-polymorphisms, with a dysregulated Th1/Th2 pathway involved in inflammation, fibrosis, and autoimmunity. Identification of these subsets of patients could provide a focused population for rational selection of therapeutic options targeting these distinct pathways involved in the pathogenesis of SSc and other fibrotic disorders.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Gourh had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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