A replication study confirms the association of *TNFSF4 (OX40L)* polymorphisms with systemic sclerosis in a large European cohort

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► Additional data (supplementary tables 1–6 and supplementary figures 1–5) are published online only. To view these files please visit the journal online (http://ard.bmj.com).

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ABSTRACT

Objectives The aim of this study was to confirm the influence of *TNFSF4* polymorphisms on systemic sclerosis (SSc) susceptibility and phenotypic features.

Methods A total of 8 European populations of Caucasian ancestry were included, comprising 3014 patients with SSc and 3125 healthy controls. Four genetic variants of *TNFSF4* gene promoter (rs1234314, rs844644, rs844648 and rs12039904) were selected as genetic markers.

Results A pooled analysis revealed the association of rs1234314 and rs12039904 polymorphisms with SSc (OR 1.15, 95% CI 1.02 to 1.31; OR 1.18, 95% CI 1.08 to 1.29, respectively). Significant association of the four tested variants with patients with limited cutaneous SSc (lcSSc) was revealed (rs1234314 OR 1.22, 95% CI 1.07 to 1.38; rs844644 OR 0.91, 95% CI 0.83 to 0.99; rs844648 OR 1.10, 95% CI 1.01 to 1.20 and rs12039904 OR 1.20, 95% CI 1.09 to 1.33). Association of rs1234314, rs844648 and rs12039904 minor alleles with patients positive for anti-centromere antibodies (ACA) remained significant (OR 1.23, 95% CI 1.10 to 1.37; OR 1.12, 95% CI 1.01 to 1.25; OR 1.22, 95% CI 1.07 to 1.38, respectively). Haplotype analysis confirmed a protective haplotype associated with SSc, lcSSc and ACA positive subgroups (OR 0.88, 95% CI 0.82 to 0.96; OR 0.88, 95% CI 0.80 to 0.96; OR 0.86, 95% CI 0.77 to 0.97, respectively) and revealed a new risk haplotype associated with the same groups of patients (OR 1.14, 95% CI 1.03 to 1.26; OR 1.20, 95% CI 1.08 to 1.35; OR 1.23, 95% CI 1.07 to 1.42, respectively).

Conclusions The data confirm the influence of *TNFSF4* polymorphisms in SSc genetic susceptibility, especially in subsets of patients positive for lcSSc and ACA.

INTRODUCTION

Systemic sclerosis (SSc) is a connective tissue disorder characterised by fibrosis, vascular damage and immune imbalance. This pathology has a complex polygenic aetiology and variable clinical manifestations. Patients with SSc are commonly classified in two major subgroups: limited cutaneous systemic sclerosis (lcSSc) and diffuse cutaneous systemic sclerosis (dcSSc).[1] Autoantibody status, especially anti-centromere antibodies (ACA) and anti-topoisomerase antibodies (ATA), is clinically used as prognostic biomarker.[1]

Familial clustering and ethnic influences support the genetic component of this disease.[2] Initially, only major histocompatibility complex (MHC) genes were firmly associated with SSc.[2] Nevertheless, recently a number of candidate genes such as *STAT4*, *BANK1* or *IRF5*, have been related to SSc genetic predisposition in independent populations by well powered studies.[3] Hypothesis free approaches such as genome-wide association studies, have lately confirmed the role of *MHC*, *IRF5* and *STAT4* and uncovered new SSc susceptibility loci, such as *CD247*.[4 5]

In this line of research, four *TNFSF4* promoter single nucleotide polymorphisms (SNPs) rs1234314, rs844644, rs844648 and rs12039904 were recently implicated in susceptibility to SSc in a Caucasian American population (composed of 1059 patients with SSc and 698 healthy controls).[6] Interestingly, the *TNFSF4* gene, which encodes OX40L, is considered as a potential autoimmunity candidate gene. OX40L is expressed on activated antigen presenting cells and endothelial cells in acute

inflammation. Furthermore, it enhances B cell proliferation and differentiation,[7] and its binding to OX40 (CD134) promotes proliferation and survival of T cells.[8] All these processes could play an important role in loss of immune tolerance and pathology as observed in SSc.

On this basis, the aim of this study was to replicate the association of *TNFSF4* gene promoter polymorphisms with SSc through a large association study in eight independent European populations of Caucasian ancestry, in order to confirm the implication of *TNFSF4* gene in SSc genetic susceptibility and phenotypic features.

PATIENTS AND METHODS

Patients

A total of 3014 cases and 3125 controls from 8 European Caucasian cohorts (Spain, Germany, The Netherlands, Belgium, Italy, Sweden, Norway and UK) were included in this study. Patients with SSc were diagnosed accordingly with the 1980 American College of Rheumatology classification criteria for SSc,[9] and were subdivided into those with lcSSc and dcSSc as defined by LeRoy *et al*.[10]

The following clinical data was collected for ascertainment of clinical phenotype of patients with SSc: age, gender, disease duration and presence of SSc specific autoantibodies, ATA and ACA. Clinical subtype information was available for 82% of the patients, and autoantibody status was available for 74% of the patients. The control population consisted of unrelated healthy individuals recruited in the same geographical regions as patients with SSc, matched by age, sex and ethnicity. The local ethical committees at all participating centres approved the study. Patients and controls were included in the study after written informed consent was obtained.

TNFSF4 polymorphisms genotyping

SNPs rs1234314, rs844644, rs844648 and rs12039904 (tag-SNP of rs2205960 SNP) were genotyped using TaqMan SNP genotyping assays in a 7900HT Real-Time PCR System from Applied Biosystems (Foster City, California, USA). The genotyping call rate was over 93% in all cases and controls included.

Statistical analysis

Association was calculated by 2×2 contingency tables and Fisher's exact test or χ^2 when necessary, obtaining p values, OR and 95% CI using PLINK (V.1.06; http://pngu.mgh.harvard.edu/purcell/plink/). p Values below 0.05 after Benjamini and Hochberg False Discovery Rate Method correction were considered as statistically significant. Hardy–Weinberg equilibrium (HWE) was tested for all SNPs at significance level=0.01.

Haplotypes were constructed using PLINK (V.1.06) and Haploview V.4.2 (http://www.broadinstitute.org/haploview/haploview). Haplotypes having a frequency <5% in control groups were excluded for the analysis. Haplotype p values were corrected using Bonferroni correction. Meta-analysis was carried out by PLINK (V.1.06) and StatsDirect (V.2.6.6; StatsDirect, Altrincham, UK) in the case of haplotypes. Homogeneity among cohorts was calculated using the Breslow–Day method, and OR calculation was performed under fixed effects model (Mantel–Haenszel) or random effects (DerSimonian–Laird) when necessary.

The power of the study for the whole set of patients and controls reached over 98% (Power Calculator for Genetic Studies 2006[11]).

RESULTS

Analysis of TNFSF4 promoter polymorphisms

The allelic frequencies of the four SNPs tested were similar to those reported for Caucasian populations in previous studies[6 12 13] and the international HapMap Project (http://hapmap.ncbi.nlm.nih.gov/). In addition, the genotypic distribution of healthy controls and SSc cases was in HWE for all SNPs.

Table 1 describes allelic distribution of the four SNPs in the pooled analysis, and supplementary tables 1–4 contain detailed data for each population. Pooled analysis of rs1234314 SNP showed statistically significant association of the G allele with SSc (p=0.03, OR 1.15, 95% CI 1.02 to 1.31), with the subset of patients with lcSSc (p=0.003, OR 1.22, 95% CI 1.07 to 1.38) and with patients positive for ACA (p=2.51E-04, OR 1.23, 95% CI 1.10 to 1.37) (table 1 and supplementary figure 1). The association of this genetic marker with lcSSc remained significant after the comparison of this subgroup of patients with those having dcSSc (p=0.01, OR 0.85, 95% CI 0.75 to 0.96, data not shown). Pooled analysis revealed a significant protective association of rs844644 minor allele with lcSSc (p=0.03, OR 0.91, 95% CI 0.83 to 0.99) (table 1, supplementary figure 2). Similarly, the rs844648 A allele showed a significant association with susceptibility to lcSSc and ACA positive subgroups (p=0.04, OR 1.10, 95% CI 1.01 to 1.20; p=0.04, OR 1.12, 95% CI 1.01 to 1.25, respectively) (table 1, supplementary figure 3). Pooled analysis revealed a strong association of rs12039904 T allele with patients with SSc (p=1.53E-04, OR 1.18, 95% CI 1.08 to 1.29), with patients in the lcSSc subgroup (p=2.81E-04, OR 1.20, 95% CI 1.09 to 1.33) and patients in the ACA-positive subgroup (p=2.09E-03, OR 1.22, 95% CI 1.07 to 1.38) (table 1, supplementary figure 4).

TNFSF4 haplotype analysis

Haplotypes represented in over 5% of the healthy controls in any of the eight populations considered, were selected for pooled analysis (table 2 and supplementary table 5). Linkage disequilibrium patterns were tested in each of the eight cohorts analysed (supplementary table 6). Only two haplotypes reached significant association with SSc, CAGC (p=2.30E-03, OR 0.88, 95% CI 0.82 to 0.96) and GCAT (p=9.10E-03, OR 1.14, 95% CI 1.03 to 1.26) (supplementary table 5) (the order of the SNPs is rs1234314-rs844644-rs844648-rs12039904). Interestingly, the CAGC haplotype is composed by the protective alleles of all the tested SNPs while the GCAT haplotype harbours all the risk alleles. The association of CAGC and GCAT haplotypes with SSc clinical features remained significant for patients in the lcSSc (p=6.8E-03, OR 0.88, 95% CI 0.80 to 0.96; p=1.3E-03, OR 1.20, 95% CI 1.08 to 1.35, respectively, data not shown) and ACA-positive subsets (p=0.01, OR 0.86, 95% CI 0.77 to 0.97; p=3.7E-03, OR 1.23, 95% CI 1.07 to 1.42, respectively, data not shown).

DISCUSSION

TNFSF4 polymorphisms have been related to susceptibility for different autoimmune diseases including SSc.[6 14] With the aim of validating the initially reported association of *TNFSF4* gene in SSc, we conducted a large case-control study and a pooled analysis in eight independent European populations of Caucasian ancestry.

In accordance with the report by Gourh *et al*,[6] our study supports the implication of *TNFSF4* gene promoter polymorphisms in SSc genetic predisposition. Stratification by SSc clinical subtype or autoantibody status confirmed the significant association of the *TNFSF4* variants with the patients in the lcSSc subset and

ACA-positive subgroup but not with patients in the dcSSc or ATA-positive subsets. Nevertheless, the risk or protective directions in the associations were consistent with those reported by Gourh *et al*.[6]

Similarly, haplotype pooled analysis results obtained in the present study keep in with the findings from Gourh *et al*.[6] The most represented haplotype in both reports are equivalent, appear in similar frequency and have a protective effect. Nevertheless, in our study the opposite haplotype GCAT, which could not be observed in the previous study, showed a significant risk association with SSc.

Interestingly, previous findings in systemic lupus erythematosus revealed the existence of equivalent protective and risk haplotypes to the ones reported in this study. Moreover, functional data showed that the risk haplotype produced an increased level of *TNFSF4* transcript (compared to the protective haplotype), and a higher surface expression of OX40L in lymphoblastoid cell lines and peripheral blood lymphocytes after activation. This overexpression seems to be related to the destruction of the DNA binding site for the transcriptional repressor E4BP4 (with a role in the survival of early B cell progenitors).[12] Thus, the *TNFSF4* risk haplotype associated with SSc and producing higher levels of OX40L might be implicated in the pathogenic mechanisms of SSc, by the alteration of regulatory processes controlling B and T cell proliferation and differentiation, leading to autoantibody production and tissue damage.[7 8 14–16] Further

Table 1 Pooled analysis of *TNFSF4* promoter genetic variants

SNP (minor/major alleles), chromosome position (bp)		N	MAF	P_{MH}	P_{FDR}	OR (95% CI)
rs1234314 (G/C), 171444015	Controls	2920	0.41			
	SSc	2856	0.44	0.03*†	–	1.15 (1.02 to 1.31)
	lcSSc	1608	0.46	0.003*‡	–	1.22 (1.07 to 1.38)
	dcSSc	724	0.42	0.75§	0.84	1.02 (0.91 to 1.15)
	ACA+	828	0.46	2.51E-04¶	0.001	1.23 (1.10 to 1.37)
	ATA+	519	0.42	0.23**	0.43	1.08 (0.95 to 1.24)
rs844644 (A/C), 171476118	Controls	2946	0.47			
	SSc	2912	0.45	0.049†	0.07	0.93 (0.86 to 1.00)
	lcSSc	1653	0.45	0.03‡	0.04	0.91 (0.83 to 0.99)
	dcSSc	743	0.46	0.84§	0.84	0.99 (0.88 to 1.11)
	ACA+	856	0.44	0.049¶	0.049	0.90 (0.80 to 1.00)
	ATA+	533	0.46	0.33**	0.43	0.94 (0.82 to 1.07)
rs844648 (A/G), 171490486	Controls	2977	0.43			
	SSc	2940	0.44	0.07†	0.07	1.07 (1.00 to 1.15)
	lcSSc	1673	0.45	0.04‡	0.04	1.1 (1.01 to 1.20)
	dcSSc	742	0.42	0.69§	0.84	0.98 (0.87 to 1.10)
	ACA+	860	0.45	0.04¶	0.049	1.12 (1.01 to 1.25)
	ATA+	529	0.42	0.74**	0.74	1.02 (0.89 to 1.17)
rs12039904 (T/C), 171478896	Controls	2991	0.23			
	SSc	2894	0.26	1.53E-04†	6.12E-04	1.18 (1.08 to 1.29)
	lcSSc	1639	0.26	2.81E-04‡	5.61E-04	1.20 (1.09 to 1.33)
	dcSSc	735	0.24	0.3§	0.84	1.07 (0.94 to 1.23)
	ACA+	840	0.26	2.09E-03¶	4.17E-03	1.22 (1.07 to 1.38)
	ATA+	523	0.24	0.15**	0.43	1.12 (0.96 to 1.31)

Controls are used as reference for all comparisons.
*DerSimonian–Laird random effects model p value.
†Breslow–Day rs1234314 p=0.01; rs844644 p=0.23; rs844648 p=0.52; rs12039904 p=0.50.
‡Breslow–Day rs1234314 p=0.08; rs844644 p=0.52; rs844648 p=0.33; rs12039904 p=0.48.
§Breslow–Day rs1234314 p=0.61; rs844644 p=0.49; rs844648 p=1.00; rs12039904 p=0.38.
¶Breslow–Day rs1234314 p=0.29; rs844644 p=0.79; rs844648 p=0.94; rs12039904 p=0.74.
**Breslow–Day rs1234314 p=0.41; rs844644 p=0.73; rs844648 p=0.71; rs12039904 p=0.56.
ACA, anti-centromere antibodies; ATA, anti-topoisomerase antibodies; chromosome position, position in chromosome 1; dcSSc, diffuse cutaneous systemic sclerosis; lcSSc, limited cutaneous systemic sclerosis; MAF, minor allele frequency; P_{FDR}, corrected p value using Benjamini and Hochberg False Discovery Rate; P_{MH}, allelic Mantel–Haenszel fixed effects model p value; SSc, systemic sclerosis.

Table 2 Pooled analysis of *TNFSF4* haplotypes in patients with systemic sclerosis and controls

(2n cases/2n controls)	Haplotype	Cases (%)	Controls (%)	P_{MH}	OR (95% CI)
Pooled (5222/5296)	CAGC	40.49	43.19	2.30E-03*	0.88 (0.82 to 0.96)
	CCGC	10.23	10.04	0.47†‡	1.09 (0.86 to 1.39)
	GAGC	4.43	3.75	0.08§	1.20 (0.98 to 1.47)
	GCAC	16.71	16.68	0.53¶	0.97 (0.87 to 1.07)
	GCAT	21.54	19.33	9.10E-03**	1.14 (1.03 to 1.26)
	Others	6.60	7.01	0.67*††	0.94 (0.73 to 1.23)

The order of the SNPs is rs1234314-rs844644-rs844648-rs12039904.
*Breslow–Day p=0.22.
†DerSimonian–Laird random effects model p value.
‡Breslow–Day p=0.004.
§Breslow–Day p=0.62.
¶Breslow–Day p=0.16.
**Breslow–Day p=0.24.
††Breslow–Day p=0.01.
PMH, allelic Mantel–Haenszel fixed effects model p value.

studies are necessary to elucidate the exact molecular mechanisms by which OX40L is implicated in SSc pathogenesis and more precisely how it can lead to the development of lcSSc and ACA production.

In summary, our results confirm the implication of *TNFSF4* promoter polymorphisms in SSc susceptibility, especially in patients in the lcSSc and ACA-positive subgroups. These findings together with previous genetic and functional studies suggest *TNFSF4* as an interesting and consistent genetic factor for SSc and other autoimmune diseases and may open new opportunities for SSc treatment.

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