Confirmation of *CCR6* as a risk factor for anti-topoisomerase I antibodies in systemic sclerosis

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ABSTRACT

Objective. *The current knowledge of the influence of systemic sclerosis (SSc) risk loci in the clinical sub-phenotypes is still limited. The main limitation lies in the low frequency of some sub-phenotypes which could be solved by replication studies in independent cohorts and meta-analysis between studies. In this regard,* CCR6 *gene variants have been recently associated with anti-topoisomerase I positive (ATA+) production in SSc patients in a candidate gene study. This gene has been proposed to have a critical role in IL-17-driven autoimmunity in human diseases.*

Methods. *In order to confirm the association between* CCR6 *and ATA+ SSc patients, we performed an independent replication study in populations of European ancestry. We studied two* CCR6 *genetic variants (rs968334 and rs3093024) in a total of 901 ATA+ SSc cases, 3,258 ATA- SSc cases and 7,865 healthy controls and compared allelic frequencies for those SNPs in ATA+ SSc with healthy controls and also with ATA- SSc patients.*

Results. *The comparison performed between ATA+ SSc patients and healthy controls showed significant association with SNP rs968334 ($p=4.88 \times 10^{-2}$, OR=1.11). When we compared ATA+ SSc cases with ATA- SSc, both SNPs, rs3093024 and rs968334, showed significant associations ($p=2.89 \times 10^{-2}$, OR=1.13; $p=1.69 \times 10^{-2}$, OR=1.15). Finally, in order to increase even more sample size and statistical power, we meta-analysed our study with the previous reported and found a significant association between SNP rs3093024 and ATA+ SSc patients ($p=1.00 \times 10^{-4}$, OR=1.16) comparing with healthy controls.*

Conclusion. *Our work confirms the association of CCR6 gene and ATA+ SSc patients.*

Introduction

Systemic sclerosis (SSc), also known as scleroderma, is an inflammatory autoimmune disease characterised by fibrosis of the skin and internal organs, vascular damage and altered immune responses with autoantibody production (especially anticentromere (ACA) and antitopoisomerase I (ATA) autoantibodies) (1). As a complex disease, SSc is caused by a combination of genetic and environmental factors (1). The genetic component has been widely explored in recent years and the number of new susceptibility loci associated with SSc has remarkably grown (2-4). Unfortunately, the knowledge of the genetic risk loci associated with SSc clinical sub-phenotypes is still limited. In particular, SSc with antitopoisomerase I positive autoantibodies (ATA^+) is the sub-phenotype with the lowest frequency among SSc patients, representing around 20% of the total (5). Until now only a few numbers of non-HLA loci have been associated with SSc ATA^+ subgroup (*NOTCH4* and *BANK1*) (6, 7) and the most plausible reason for this may be the small sample size in ATA subgroup. In these cases the use of replication studies in independent cohorts and meta-analysis between studies could solve this limitation.

In this regard, ATA+ SSc patients has been recently found associated with

two polymorphisms (rs3093023 and rs10946216) located in *CCR6* gene through a candidate gene study (8). This gene has a great relevance in autoimmunity because it encodes a chemokine receptor with an important role in B cell differentiation and migration during inflammatory and immunological responses (9). In addition, this gene has been associated with other autoimmune diseases like rheumatoid arthritis and Crohn's disease (10, 11) although not with all (12). Interestingly, a correlation has been found between one *CCR6* functional variant (CCR6DNP) and interleukin 17 (IL-17) serum levels in rheumatoid arthritis patients (11).

Despite significant association results found by Koumakis *et al*. in a candidate gene study, this work needs to be validated by an independent study. Thereby, in order to confirm *CCR6* as an ATA+ SSc susceptibility locus, we designed an independent replication study in populations with European origin and a meta-analysis with the previous data published (8).

Material and methods

Subjects

Our replication study included a total of 901 ATA+ SSc cases, 3,258 ATA- SSc cases and 7,865 healthy controls from nine cohorts of European descent (Spain, Germany, The Netherlands, USA Italy and The UK) (4). SSc cases were classified based on their skin involvement into limited cutaneous SSc (lcSSc) or diffuse cutaneous SSc (dcSSc) according to the criteria by LeRoy *et al*. (13). The clinical phenotype of patients with SSc was determined by the following clinical data: age, gender and presence of SSc-associated autoantibodies (ACA and ATA) (14). The control population cohort consisted of unrelated healthy individuals and was recruited in the same geographical regions as SSc patients.

Local ethics committees from all participating centres approved the study: Comité de Bioética del Consejo Superior de Investigaciones Científicas, Comitato Etico Azienda Ospedaliera Universitaria Integrata di Verona, Ethics Committee of the University Erlangen-Nuremberg, Local Ethics Committee of the Radboud University Nijmegen Medical Centre, Local Research Ethics Committee at Glasgow Royal Infirmary, Ethics Review Board of the Ruhr University Bochum, U.O. Comitato di Etica e Sperimentazione Farmaci Fondazione IRCCS Ca' Granda - Ospedale Maggiore Policlinico di Milano, Ethik-Kommission der MHH, Ethics Committee of the University of Cologne, Ethics Committee of the Charité University Hospital, Centre for Biomedical Ethics Katholieke Universiteit Leuven and Manchester University Research Ethics Committee. Both patients and controls were included in the study after written informed consent.

Genotyping and statistical analysis

In our study we analysed genotype data of 2 SNPs located in *CCR6* gene: rs3093024 and rs968334. Our Spain I, Netherlands I and US cohort genotyped data was obtained from Radstake *et al*. (4) while the rest of the cohorts (Spain II, Germany, Netherlands II, Italy and UK) were genotyped by TaqMan SNP® genotyping assays (Applied Biosystems) in a LightCycler®480 SNP Genotyping System from Roche. Samples that overlapped with the previous study of Koumakis *et al*. were excluded from the analyses (8). All data was quality filtered as described Radstake *et al*. (4). Cochran-Mantel-Haenszel meta-analysis was performed to control for the differences among populations as implemented in PLINK software (15). Heterogeneity between cohorts was tested using the Breslow-Day test and represented by forest plot. To test for associations, *P*-values were obtained by performing 2×2 contingency tables and χ^2 test and/or Fisher's exact test, when appropriate. ORs and 95% CI were calculated according to Woolf's method. Meta-analyses were performed by Cochran-Mantel-Haenszel analysis with StatsDirect v.2.4.6 (Altrincham, UK). *P*-values <0.05 were considered as statistically significant.

Table I. Pooled analysis of SNPs rs3093024 and rs968334 in samples with European origin.

SNP	1/2	Subgroup	n	P_{MH}	OR (CI95%)
rs3093024	A/G	Controls	7,865		
		ATA+ SSc	901	0.09	1.09 (1.0-1.2)
		ATA- SSc[1]	3,258	**2.89E-02**	1.13 (1.0-1.2)
rs968334	T/C	Controls	7,865		
		ATA + SSc	901	**4.88E-02**	1.11 (1.0-1.2)
		ATA- SSc[1]	3,258	**1.68E-02**	1.14 (1.0-1.3)

All the comparisons were made comparing with healthy controls.[1]Comparison were made comparing ATA+ SSc individuals with ATA- SSc individuals. [2]Replication phase I +II + Koumakis *et al*. (MAF, minor allele frequency; OR: odds ratios; CI: confidence interval; MH: Cochran-Mantel-Haenszel; SSc: systemic sclerosis; ATA: anti-topoisomerase I antibodies).

Results

In order to confirm the association of *CCR6* with ATA+ SSc patients, we selected 2 SNPs: rs3093024 in high linkage disequilibrium with the SNPs found previously associated with this phenotype (8) (rs3093023 r^2=1; rs10946216 r^2=0.96), and one SNP of CCR6DNP functional variant (rs968334) (11).

Our results obtained in replication phase showed significant associations with both SNPs (Table I). SNP rs3093024, closely related with the previous association report, showed significant results in the comparison between ATA+ and ATA- SSc patients (*P*=2.89 x 10^{-2}, OR=1.13). In contrast, SNP rs968334, part of CCR6DNP functional variant, showed significant association with ATA+ SSc patients when we compared with healthy controls (*P*=4.88 x 10^{-2}, OR=1.11) and with ATA- SSc patients (*P*=1.69 x 10^{-2}, OR=1.14) (Table I). Besides, to determine if the signal detected in ATA is a consequence of these autoantibodies or it is due to the relationship of ATA subgroup with diffuse SSc or lung fibrosis, we evaluated in our replication study the association of these SNPs with these subsets (Supplementary Table). These analyses showed that the most significant association with *CCR6* variants is with ATA subgroup.

Table II. Power calculations of the different stages of the study.

Group	n		OR 1.3 MAF						OR 1.2 MAF						rs3093024 OR / MAF
	SSc ATA+	Controls	0.4	0.3	0.2	0.2	0.1	0.1	0.4	0.3	0	0.2	0	0.1	**1.17 / 0.44**
Koumakis *et al*.	648	6.912	100	100	99	96	88	61	93	90	81	72	56	32	85
Replication Phase	889	7.865	100	100	100	99	96	75	98	97	91	84	70	42	93
Replication Phase + Koumakis *et al*.	1.537	14.777	100	100	100	100	100	94	100	100	99	97	90	64	**99**

(MAF, minor allele frequency; OR: odds ratios; SSc: systemic sclerosis; ATA: anti-topoisomerase antibodies).

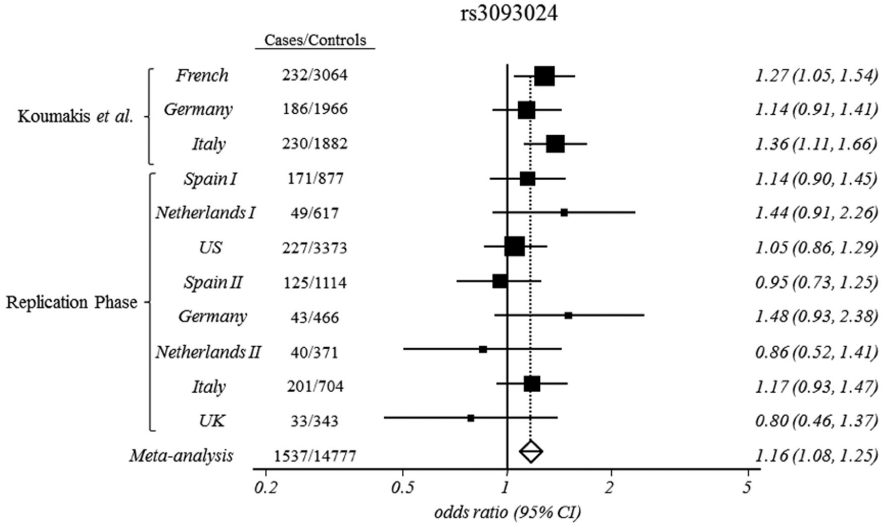

Fig. 1. Forest plot for the meta-analysis of the rs3093024 CCR6 genetic variant in ATA+ SSc patients.

Supplementary Table. Replication study results of rs3093024 and rs968334 CCR6 gene variants in SSc samples and its subsets.

SNP	1/2	Subgroup	Replication phase n	P_{MH}	OR (CI95%)
rs3093024	A/G	Controls	7.865		
		SSc	7.865	0,824	0.99 (0.9-1.1)
		ISSc	2.674	0,775	0.99 (0.9-1.1)
		dSSc	1.262	0,590	1.02 (0.9-1.1)
		ACA+	1.581	0,742	0.98 (0.9-1.1)
		ATA +	900	0,090	1.09 (1.0-1.2)
		ATA -[1]	3.258	**2,89E-02**	1.13 (1.0-1.2)
		PF+[¥]	749	0,158	1.08 (1.0-1.2)
		PF-[2]	1.859	0,070	1.12 (1.0-1.2)
rs968334	T/C	Controls	7.865		
		SSc	4.528	0,781	0.99 (0.9-1.1)
		ISSc	2.674	0,796	0.99 (0.9-1.1)
		dSSc	1.262	0,699	1.02 (0.9-1.1)
		ACA+	1.581	0,742	0.99 (0.9-1.1)
		ATA +	900	**4,88E-02**	1.11 (1.0-1.2)
		ATA -[1]	3.258	**1,68E-02**	1.14 (1.0-1.3)
		PF+[¥]	749	0,157	1.08 (1.0-1.2)
		PF-[2]	1.859	0,157	1.09 (1.0-1.2)

All the comparisons were made comparing with healthy controls. [1]Comparison were made comparing ATA+ SSc individuals with ATA- SSc individuals. [2] Comparison were made comparing PF+ SSc individuals with PF- SSc individuals. [¥] USA cohort was removed from analysis because their diagnosis criteria of pulmonary fibrosis were different from European diagnosis criteria (Controls n=1544). (MAF: minor allele frequency; OR: odds ratios; CI: confidence interval; MH: Cochran-Mantel-Haenszel; SSc: systemic sclerosis; lSSc: limited systemic sclerosis; dSSc: diffused systemic sclerosis; ACA: anti-centromere B antibodies; ATA: anti-topoisomerase antibodies; PF: pulmonary fibrosis).

In order to increase sample size and statistical power, we meta-analysed our data with the previous reported study (Table II). Previously to the analysis, all samples suspected to be overlapping between studies were excluded from our study. Besides, only one SNP was included in both studies, SNP rs3093024, thus meta-analysis was focused in it. The representation by forest plot of the eleven cohorts included in meta-analysis in the comparison of ATA+ SSc patients and healthy controls showed no statistically significant genetic heterogeneity between them (*P* Breslow-Day=0.3965) (Fig. 1). Furthermore, the statistical power reached in the meta-analysis was 99% (OR 1.16, MAF 0.43, at the 5% significant level) according to Power Calculator for Genetic Studies 2006 software (16). Thereby the results obtained in meta-analysis showed significant association with ATA+ SSc patients (P=1.0 x 10^{-4}, OR=1.16 (95% CI 1.08–1.25)) when we compared with healthy controls.

Discussion

In the present work, we performed an independent replication study in populations of European ancestry and a meta-analysis with the previously published data on *CCR6*. Our work confirms the association previously observed between *CCR6* and ATA+ SSc patients with the largest cohort of ATA+ patients used until now (8). The current work support the need of larger samples sizes to uncover SSc risk loci in the clinical sub-phenotypes.

The most significant association found in our replication was detected in the comparison between ATA+ and ATA- SSc patients in SNP rs968334. This SNP is part of functional CCR6DNP variant which correlated with the expression

level of *CCR6* and was associated with the presence of interleukin-17 (IL-17) in the sera of rheumatoid arthritis patients (11). In addition, we also showed significant association with this SNP in the comparison between ATA+ SSc patients and healthy controls. Unfortunately, this SNP had not been analysed in the previous work and for this reason has not been included in meta-analysis. Thus, future studies will be necessary to determine the relevance of CCR6DNP functional variant in SSc patients. On the other hand, SNP rs3093024 also showed significant genetic association when comparing ATA+ and ATA- SSc patients. This SNP is located in intron 1 of *CCR6* gene and had been previously associated with rheumatoid arthritis (11). SNP rs3093024 is in high linkage disequilibrium with SNP rs3093023 (r^2=1) previously associated with ATA+ SSc patients by Koumakis *et al*. (8) and with functional CCR6DNP variant (r^2>0.8). Besides, the meta-analysis of our study with previous report also detected significant association with SNP rs3093024. Altogether, these results support the previous association of *CCR6* and ATA+ SSc patients.

The relevance of anti-topoisomerase autoantibodies lies in the correlation found between its levels and skin score, disease severity and disease activity (17). Moreover, it has been proposed that the *CCR6* gene is involved in IL-17-driven autoimmunity in human diseases (11). This gene encoded a specific marker for Th17 cells (18), which appear at sites of inflammation and act attracting other T-helper cells to the inflammatory site. Besides, they are characterised by the production of interleukin-17 (IL-17) which has been found to be increased in patients with SSc (19). Furthermore, serum levels of IL-17 have been also correlated with disease severity (20). In summary, both ATA and IL-17 levels in SSc patients have been correlated with severity but to uncover the implication of *CCR6* gene in the severity of these patients will be necessary future studies.

In conclusion, we confirm the association of *CCR6* gene and SSc patients with ATA+ autoantibodies. The goal of our study was to combine two independent studies reaching the highest number of ATA+ SSc patients analysed at the moment which has allowed us to confirm with high statistical power the previously described association.

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