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


Authors’ affiliations on page S-34.

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Key words: CCR6, anti-topoisomerase antibody, systemic sclerosis, genetic association, SNP

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 autoimmune disease.

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 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 x 10², OR=1.11). When we compared ATA+ SSc cases with ATA- SSc, both SNPs, rs3093024 and rs968334, showed significant associations (p=2.89 x 10², OR=1.13; p=1.69 x 10², OR=1.15).

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 anticientromere (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 have 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 autoimmune immunity 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 χ² 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.

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 r²=1; rs10946216 r²=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+ SSc patients 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 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>
<thead>
<tr>
<th>SNP</th>
<th>1/2</th>
<th>Subgroup</th>
<th>n</th>
<th>P_MH</th>
<th>OR (CI95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3093024</td>
<td>A/G</td>
<td>Controls</td>
<td>7,865</td>
<td>0.09</td>
<td>1.09 (1.0-1.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATA+ SSc</td>
<td>901</td>
<td></td>
<td>2.89E-02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATA- SSc¹</td>
<td>3,258</td>
<td></td>
<td>1.13 (1.0-1.2)</td>
</tr>
<tr>
<td>rs968334</td>
<td>T/C</td>
<td>Controls</td>
<td>7,865</td>
<td></td>
<td>4.88E-02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATA+ SSc</td>
<td>901</td>
<td></td>
<td>1.11 (1.0-1.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATA- SSc¹</td>
<td>3,258</td>
<td></td>
<td>1.14 (1.0-1.3)</td>
</tr>
</tbody>
</table>

All the comparisons were made comparing with healthy controls. Comparison were made comparing ATA+ SSc individuals with ATA- SSc individuals. 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).
**CCR6 is a genetic risk for anti-topoisomerase I positive antibodies in SSc / E. Ochoa et al.**

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 ($P_{\text{Breslow-Day}}=0.3965$) (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 ($P=1.0 \times 10^{-4}$, 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

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

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>OR 1.3 MAF</th>
<th>OR 1.2 MAF</th>
<th>rs3093024 OR / MAF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SSc Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.4</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Koumakis et al.</td>
<td>648</td>
<td>7.865</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Replication Phase</td>
<td>889</td>
<td>7.865</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Replication Phase + Koumakis et al.</td>
<td>1.537</td>
<td>14.777</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

(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.

<table>
<thead>
<tr>
<th>SNP</th>
<th>1/2</th>
<th>Subgroup</th>
<th>n</th>
<th>$P_{\text{meta}}$</th>
<th>OR (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3093024</td>
<td>A/G</td>
<td>Controls</td>
<td>7.865</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>SSc</td>
<td>7.865</td>
<td>0.824</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ISSc</td>
<td>2.674</td>
<td>0.775</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dSSc</td>
<td>1.262</td>
<td>0.590</td>
<td>1.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ACA+</td>
<td>1.581</td>
<td>0.742</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATA+</td>
<td>900</td>
<td>0.090</td>
<td>1.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATA-</td>
<td>3.258</td>
<td>2.89E-02</td>
<td>1.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PF+</td>
<td>749</td>
<td>0.158</td>
<td>1.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PF-</td>
<td>1.859</td>
<td>0.070</td>
<td>1.12</td>
</tr>
<tr>
<td>rs968334</td>
<td>T/C</td>
<td>Controls</td>
<td>7.865</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>SSc</td>
<td>4.528</td>
<td>0.781</td>
<td>0.99</td>
</tr>
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<td></td>
<td>ISSc</td>
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<td>0.796</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dSSc</td>
<td>1.262</td>
<td>0.699</td>
<td>1.02</td>
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<td></td>
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<td>ACA+</td>
<td>1.581</td>
<td>0.742</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATA+</td>
<td>900</td>
<td>4.88E-02</td>
<td>1.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ATA-</td>
<td>3.258</td>
<td>1.68E-02</td>
<td>1.14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PF+</td>
<td>749</td>
<td>0.157</td>
<td>1.08</td>
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<tr>
<td></td>
<td></td>
<td>PF-</td>
<td>1.859</td>
<td>0.157</td>
<td>1.09</td>
</tr>
</tbody>
</table>

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. 3 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; ISSc: limited systemic sclerosis; dSSc: diffused systemic sclerosis; ACA: anti-centromere B antibodies; ATA: anti-topoisomerase antibodies; PF: pulmonary fibrosis).
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 (r²=1) previously associated with ATA+ SSc patients by Koumakis et al. (8) and with functional CCR6DNP variant (r²>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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