

No evidence of association of *ATP8B4* F436L missense variant in a large systemic sclerosis cohort

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Over the past seven years our knowledge of the systemic sclerosis (SSc) genetic component has considerably increased, mainly thanks to large genetic studies including genome wide association studies (GWASs) and ImmunoChip analysis (1-3). However, there is still a large portion of the SSc heritability that remains unexplained, as for most complex traits (4). One of the hypotheses proposed to explain the missing heritability for complex diseases involves rare and low-frequency variants. These types of genetic variations are not well covered by GWAS, which are mainly focused on common variants. However next-generation sequencing technologies, like whole exome sequencing (WES), have rapidly overcome this issue. In this regard, Gao *et al.* performed for the first time WES on SSc and reported a novel gene, *ATP8B4*, as a risk factor for the disease (5). They suggested a missense rare variant (F436L [rs55687265]) as a potential causal variant for the association signal in *ATP8B4*. Therefore our aim was to further evaluate the reported signal of association taking advantage of our large SSc cohorts.

ATP8B4 rs55687265 rare variant was genotyped in six independent case-control sets of European ancestry reaching a total of 7,426 SSc patients and 13,087 healthy controls (Suppl. Table 1). All SSc patients fulfilled previously described classification criteria for the disease (3). First, we performed association analyses to test whether *ATP8B4* rs55687265 was associated with SSc susceptibility in each of the cohorts included in the present study (Supp Methods). A trend towards association was observed for the Spanish case-control set ($P_{\text{value}} = 0.071$, OR = 1.58) (Table 1). However, we did not observe any suggestive or significant signal of association for the remaining cohorts. We also observed opposite-direction allelic effects across the different case-control sets. The meta-analysis combining all the sample sets, which was performed using an inverse variance fixed effects model, showed no significant association with the disease ($P_{\text{meta}} = 0.484$, OR = 1.07) (Table 1 and

Supp Methods). Thus, according to our results, we did not find statistically significant differences of the *ATP8B4* rs55687265*C allele frequency between the SSc patients and controls enrolled in the study. It is noteworthy that the impact of rare variants in the development of autoimmune diseases (ADs) remains an unanswered and controversial question (6). Moreover, high throughput DNA sequencing technologies, such as WES, are substantially affected by technical artifacts, which may lead to type I errors (7). This issue becomes especially important when cases and controls have not been exactly sequenced in the same way, which leads to excessive false positive findings in detecting rare variant associations (7-9). The present study highlights the importance of validation of WES results with other sequencing methods as well as replication of the new associations in independent studies in order to detect actual disease-causing mutations.

In conclusion, this study could not replicate the association of *ATP8B4* rs55687265. However, the possible role of this gene cannot be discarded, since the present report did not attempt to evaluate whether other rare or common variants were associated with SSc susceptibility.

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TABLE

Table 1. Association analysis of *ATP8B4* F436L variant in six independent cohorts and meta-analysis.

Cohort	Minor/Major	Cases/Controls	MAF Cases	MAF Controls	P-value	OR* [CI 95%]
Spain	C/G	2,056/2,718	0.008	0.005	0.071	1.58 [0.96-2.61]
Germany	C/G	909/486	0.019	0.022	0.613	0.87 [0.50-1.50]
The Netherlands	C/G	435/783	0.013	0.007	0.185	1.78 [0.75-4.20]
Italy	C/G	1,114/980	0.006	0.011	0.096	0.56 [0.28-1.12]
UK	C/G	1,456/5,272	0.012	0.011	0.532	1.13 [0.77-1.67]
USA	C/G	1,456/2,848	0.015	0.015	0.991	1.00 [0.69-1.45]
Meta-analysis**	C/G	7,426/13,087			0.484	1.07 [0.88-1.31]

*Odds ratio for the minor allele.

**Q heterogeneity value = 0.17; I = 35.73

CI, confidence interval; MAF, minor allele frequency; OR, odds ratio.

SUPPLEMENTARY MATERIAL

Supplementary methods

1. Genotyping methods

The genotyping of *ATP8B4* rs55687265 was performed with TaqMan SNP genotyping technology (assay ID: AHI1051) in a LightCycler® 480 Real-Time PCR System (Roche Applied Science, Mannheim, Germany). Genotyping call rate was > 95%. All the samples were genotyped by TaqMan assay, with the exception of the UK and USA control cohorts. For these two control sets, genotyping data were obtained from previously published studies (WTCCC and Lung Health Study, respectively) (1-2).

2. Data analysis

All the statistical analyses were carried out with PLINK v1.07 (<http://pngu.mgh.harvard.edu/purcell/plink/>). Association test for the six cohorts were performed in each population by 2x2 contingency tables and χ^2 test. *P*-values lower than 0.05 were considered as statistically significant. The meta-analysis was performed with inverse-variance method under a fixed-effects model. Heterogeneity of the ORs across cohorts was assessed using Cochran's Q test. None of the included control sets showed significant deviation from Hardy Weinberg Equilibrium (HWE) (HWE *P*-values < 0.01 were considered to show significant deviation from equilibrium). The statistical power of the combined analysis is shown in Supp. Table 2 and was calculated according to Power Calculator for Genetic Studies 2006 software under an additive model (3).

Supplementary tables

Supp. Table 1. Study cohorts

COHORT	Patients with SSc	Controls
Spain	2,056	2,718
Germany	909	486
The Netherlands	435	783
Italy	1,114	980
UK	1,456	5,272
USA	1,456	2,848
TOTAL	7,426	13,087

Supp. Table 2. Overall statistical power of the study for a statistical significance P -value = 0.05.

	OR = 1.2	OR = 1.5	OR = 1.8	OR = 2.2	OR = 2.5	OR = 2.8
rs55687265	43%	99%	100%	100%	100%	100%

Supplementary note

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