A *MIF* promoter polymorphism is associated with the susceptibility to pulmonary arterial hypertension in diffuse cutaneous systemic sclerosis patients

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ABSTRACT

Objective: Systemic sclerosis (SSc) is a fibrotic immune-mediated disease of unknown etiology. Among its clinical manifestations, pulmonary involvement is the leading cause of mortality in SSc patients. However, the genetic factors involved in lung complication are not well-defined. We aimed to revisit the association of the *MIF* gene, which encodes a cytokine implicated in idiopathic pulmonary hypertension among other diseases, with the susceptibility and clinical expression of SSc, besides testing the association of this polymorphism with SSc-related pulmonary involvement.

Methods: A total of 4,392 SSc patients and 16,591 unaffected controls from six cohorts of European origin were genotyped for the *MIF* promoter variant rs755622. An inverse variance method was used to meta-analyze the data.

Results: A statistically significant increase of the *MIF* rs755622*C allele frequency compared to controls was observed in the subgroups of patients with diffuse cutaneous SSc (dcSSc) and with pulmonary arterial hypertension (PAH) independently (dcSSc:*P*=3.20E-2, OR=1.13; PAH:*P*=2.19E-02, OR=1.32). However, our data revealed a stronger effect size with the subset of SSc patients showing both clinical manifestations (dcSSc with PAH:*P*=6.91E-3, OR=2.05).

Conclusions: We revisited the association of the *MIF* rs755622*C allele with SSc and described a phenotype-specific association of this variant with the susceptibility to develop PAH in dSSc patients.

KEY WORDS

Systemic sclerosis, *MIF*, rs755622, Pulmonary arterial hypertension.

INTRODUCTION

Systemic sclerosis (SSc) is a complex disease of unknown etiology influenced by both genetic and environmental factors. It is characterized by a progressive tissue fibrosis along with vascular anomalies and dysfunction, and the presence of auto-antibodies directed to different cellular structures, mainly anti-topoisomerase (ATA) and anti-centromere (ACA)antibodies. Depending on the extent of the skin implication, SSc is classified as diffuse cutaneous SSc (dcSSc) or limited cutaneous SSc (lcSSc). Among the clinical manifestations, pulmonary fibrosis (PF) and pulmonary arterial hypertension (PAH) are the leading cause of death in SSc patients [1]. These pulmonary complications can be present separately, as either interstitial lung disease (ILD) or isolated PAH, or combined [2].

During the last years, great advances have been made in the determination of the genetic component of SSc [3, 4]. Nevertheless, we are still far from its complete understanding, especially with regard to pulmonary involvement, in which only few associated genes have been described [3,4].

Macrophage migration inhibitory factor (*MIF*) gene encodes a constitutively expressed protein that seems to have an important role on autoimmune and inflammatory processes. Infections, pro-inflammatory cytokines and antigen-specific activation can also lead to an increased expression of *MIF* [5,6]. The promoter region of this gene contains a single nucleotide polymorphism (SNP) at position -173 (rs755622) that has been associated with several immune-mediated diseases, including SSc and systemic lupus erythematosus (SLE) [7-9]. This SNP is in high linkage disequilibrium (LD) with a functional polymorphism, a -794 CATT (5-8) microsatellite repeat (rs5844572), also located at the promoter region of the gene [10]. Interestingly, increased MIF protein levels have been reported in patients with idiopathic pulmonary fibrosis, pulmonary hypertension, and in individuals affected by SSc-associated PAH [11-13].

In the present study, we have analyzed for the first time the possible involvement of the *MIF* rs755622 polymorphism in the susceptibility to develop PF and PAH in SSc patients.

MATERIALS AND METHODS

Samples

Overall, 4,392 SSc patients and 16,591 unaffected controls from Spain, Germany, The Netherlands, Italy, the United Kingdom, and Norway were included in the present study. The cohorts included in this study were partially overlapping with the cohorts in Bossini-Castillo *et al*. [7], based on the presence of pulmonary involvement clinical data. In total, the overlap between our global study cohort and the one included in [7] was 30%.All patients were classified as having dcSSc or lcSSc, following the criteria described by LeRoy *et al.* [14]. Patients were additionally classified accordingly with the presence or absence of ACA and ATA antibodies. PF was diagnosed by the presence of interstitial abnormalities in high-resolution computed tomography (HRCT) and force vital capacity (FVC) lower than 60%. Pulmonary hypertension was considered PAH if FVC was higher than 60 % or there was not moderate-severe extent of interstitial lung disease in HRCT. Thus, patients were defined as PAH+ if they showed a mean resting pulmonary artery pressure ≥ 25 mmHg, at the time of a right heart catheterization, pulmonary artery wedge pressure (PAWP)≤15 mmHg and FVC > 60% [15, 16]. The control population consisted of unrelated healthy individuals recruited in the same geographic regions as the SSc patients.

The local ethical committees from all the contributing centers approved the project in compliance with the Helsinki Agreement (Valle de Hebron Hospital, 12 de Octubre University Hospital, San Cecilio University Hospital, de la Santa Creui Sant Pau Hospital, Carlos Haya Hospital, San Carlos Hospital, Bellvitge University Hospital, Virgen del Rocio Hospital, San Jorge General Hospital, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinic Foundation, degli Studi di Verona University, Spedali Civili Hospital, Oslo University Hospital, Josefs-Hospital, University of Lübeck, Hannover Medical School, University of Cologne, Radboud University Nijmegen Medical Centre, VU University Medical Center, Leiden University Medical Center, Glasgow Biomedical Research Centre, Manchester University, University Medical Center

Utrecht). Patients and controls gave written informed consent for their participation in the present study.

Genotyping

DNA extraction was performed using standard methods. *MIF* rs755622 was genotyped using a TaqMan® SNP genotyping assay (assay ID: C_2213785_10) in a Light Cycler 480 Real-Time PCR System (Roche Applied Science, Mannheim, Germany). We also used available *MIF* rs755622 genotyping data from a previously published study based on the Immunochip [17], a genotyping platform that was specifically designed for the study of the genetic component of immune-mediated diseases. **Supplementary Table 1** shows the number of samples genotyped in each platform. The genotyping call rate (the fraction of called samples per SNP over the total number of samples in the dataset) was 97% for the TaqMan assay and 99% for the Immunochip data. The genotype–genotype concordance was evaluated in 1,253 samples with genotyping data from both platforms. A 100% of concordance was observed between both genotyping platforms.

Statistical methods

Using the Power Calculator for Association Studies (CaTS) software 2006 (Center for Statistical Genetics, The University of Michigan, USA), and assuming an effect size of OR=1.2, we estimated that the statistical power of the study was 99% for the overall analysis of the whole cohort (SSc cases *vs*. controls), 98% for the analysis of dcSSc and PF phenotypes, 61% for the analysis of PAH+ patients and 5% for the analysis with dcSSc patients that have PAH. Since the statistical power depends on the odd ratio (OR) of the SNP, we also calculated the statistical power for this last comparison assuming the observed effect size in this study, obtaining a 97% of statistical power. Additionally, no deviation from Hardy-Weinberg equilibrium was detected in our datasets.

All the statistical analyses were carried out with PLINK v1.07 (http://pngu.mgh.harvard.edu/purcell/plink/). Association statistics for the six cohorts were calculated in each population by 2x2 contingency tables and $\chi 2$ test. *P*-values lower than 0.05 were considered as statistically significant. The inverse variance weighted fixed-effects meta-analysis method was used for the pooled analyses.

RESULTS

We first investigated whether there were significant differences between the rs755622 allele frequencies of the whole SSc group or the different stratified groups (by clinical/autoantibody phenotypes) and those of the control set. **Supplementary Table 2** summarizes the results for the analyses of the independent cohorts and **Table 1** shows the results of the pooled analysis. As observed in **Table 1**, a significant *P*-value was obtained when the dcSSc group was compared against the control set in the meta-analysis (*P*=3.20E-2, OR=1.13, 95% CI=1.01-1.26). Moreover, there was a significant increase of the minor allele frequency in the group of SSc patients affected by PAH compared to controls (*P*=2.19E-02, OR=1.32, 95% CI=1.04-1.67). However, no association was observed between rs755622 and the PF-positive SSc group (PF+ vs. controls: *P*=0.257, PF+ vs PF-:*P*=0.737).

In order to dissect out the genetic association of rs755622*C with the SSc clinical phenotypes, we classified the patients into four additional subgroups based on the SSc subtype and the presence/absence of PAH. Then, we carried out a pooled analysis of the six cohorts by comparing these new subsets against controls (**Table 2**; see **Supplementary Table 3** for the results of each cohort independently). A considerably significant difference in the rs755622 frequencies between controls and the subgroup of patients with both dcSSc and PAH phenotypes was observed (dcSSc with PAH vs. controls: *P*=6.91E-03, OR=2.05, 95% CI= 1.30-4.05).However, no association was evident in the analysis of the other subgroups against controls (lcSSc with PAH vs. controls: *P*=0.178; dcSSc without PAH vs. controls: *P*=0.083; lcSSc without PAH vs. controls: *P*=0.436). We also did not find evidence of association between *MIF* rs755622 and SSc-related PF and dcSSc-related PF (data not shown).

DISCUSSION

This study, which comprises the largest cohort of SSc-associated PAH patients analyzed to date, clearly suggests that the *MIF* rs755622*C variant is a firm genetic risk factor for the

susceptibility to PAH in dcSSc patients. Previous reports described an association of this SNP with the diffuse form of the disease [7,8]. Consistent with this, we also observed a significant increase in the *MIF* rs755622*C frequency in the dcSSc subgroup compared to the control set. However, subphenotype analyses indicated that the significant associations detected in dSSc and the PAH+ subgroups may rely on the presence of subjects with both phenotypes in each dataset. Therefore, those carrying the *MIF* rs755622*C allele are at a higher risk of suffering dcSSc with PAH rather than PAH or dcSSc independently. The fact that the strongest effect size of *MIF* rs755622*C was observed in the subset of patients having both dcSSc and PAH (OR = 2.05) clearly supports this idea. Therefore, our findings may suggest *MIF* rs755622*C as a marker for dSSc patients at risk of developing PAH.

In our set, the percentages of PAH in the different phenotype subsets, dSSc or lSSc, were 2.55 % and 6.41% respectively. This is, 14.78% of PAH+ cases were dSSc patients and 85.22% corresponded to lSSc cases, in line with previous reports in SSc patient cohorts [18, 19]. However, as mentioned above, we only found that the association observed between *MIF* rs755622 and the PAH+ group remained significant only under a dcSSc phenotype. In this regard, gene expression patterns specific for SSc, SSc-related PF and SSc-related PAH have been recently described [20], thus indicating that each SSc phenotype may be develop under a distinct molecular environment. The high specificity of the *MIF* rs755622 association with a particular subset of SSc patients may be related to this fact.

MIF rs755622 is a promoter polymorphism that has been linked to the up regulation of the *MIF* expression in immune-mediated diseases [5]. As we mentioned before, this SNP is in high LD with the functional CATT5-8 polymorphism, and it is not still established whether rs755622 influences *MIF* promoter activity by itself or whether the observed effect may be linkage to CATT5-8. Interestingly, elevated protein levels of this gene have been detected in the sera of dcSSc patients affected by PAH [13, 21]. Moreover, Le Hiress *et al*. showed through *in vitro*

studies that MIF receptor (CD74) is overexpressed in individuals with idiopathic PAH [22]. These authors also provided evidence for the effect of a MIF antagonist and anti-CD74 neutralizing antibodies on the reversion of PH in a rat model [22].MIF is a pleotropic protein expressed in several human cell types that can act as a cytokine, hormonal and immune modulatory factor. As a cytokine, MIF induces the expression and secretion of several immune mediators, including interleukin-6 (IL-6) [23]. Increased levels of IL-6 have been described in serum and lungs of idiopathic PAH patients and those with dcSSc compared to lcSSc [24,25], and IL-6 has a proved effect on the generation and development of PAH *in vivo* [24]. Moreover, allelic combinations of SNPs in the IL-6 gene have been suggested as susceptibility factors for SSc [26]. Based on the above, we speculate that *MIF* rs755622 could be influencing PAH development in dcSSc affected individuals by promoting IL-6 secretion, which could contribute to the obstruction of small pulmonary vessels that leads to hypoxia and, eventually, to PAH.

In conclusion, here we provide novel insights into the genetic background of SSc-related PAH. We have shown that the *MIF* allele rs755622*C is associated with higher risk of being affected by this severe condition in dcSSc patients, and our results may be helpful for a better evaluation of the prognosis in SSc and for the development of more effective personalized treatments.

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DISCLOSURE STATEMENT

The authors have declared no conflicts of interest.

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