# A candidate gene study reveals association between a variant of the *SRp55* splicing factor gene and systemic sclerosis

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## Abstract

### Objective

*To examine the possible implication of the mRNA-binding protein serine/arginine protein 55 (SRp55, also known as SRSF6) rs2235611 single nucleotide polymorphism (SNP) in the genetic predisposition to systemic sclerosis (SSc) susceptibility and clinical phenotype.*

### Methods

*A total population of 872 white Italian individuals (414 SSc patients, 458 controls) was studied. SSc patients were assessed for limited and diffuse cutaneous subsets and the presence of autoantibodies, interstitial lung disease (ILD), and nailfold videocapillaroscopy (NVC) abnormalities. The SRp55 rs2235611 SNP was genotyped by TaqMan real-time PCR.*

### Results

*SRp55 rs2235611 genotype distribution and allele frequency were similar in SSc and healthy controls, though a trend toward significance was observed for genotype distribution (p=0.07). The SRp55 rs2235611 AA genotype significantly influenced the predisposition to SSc (p=0.03). The SRp55 rs2235611 A minor allele and AA genotype showed a significant risk association with susceptibility to SSc-related ILD (A allele: p=0.046; AA genotype: p=0.007). A significant association of the AA genotype with SSc late NVC pattern was also found (p=0.006). After Bonferroni correction for multiple comparisons, the risk association of the SRp55 rs2235611 AA genotype with SSc-related ILD and late NVC pattern remained significant ($p_{adj}$=0.049 and $p_{adj}$=0.042, respectively).*

### Conclusion

*The SRp55 rs2235611 AA genotype significantly influences the susceptibility to SSc, and specifically associates with the presence of SSc-related ILD and late NVC pattern. Further in-depth studies on the SRp55 gene locus will hopefully contribute to extend our knowledge of the genetic predisposition to major SSc-related manifestations such as pulmonary fibrosis and peripheral microvasculopathy.*

### Key words

systemic sclerosis, scleroderma, pulmonary fibrosis, microvasculopathy, single nucleotide polymorphism, *SRp55*

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*Received on July 9, 2021; accepted in revised form on September 13, 2021.*



*Competing interests: none declared.*

## Introduction

Systemic sclerosis (SSc, scleroderma) is an autoimmune disorder characterised by innate and adaptive immune system abnormalities leading to production of autoantibodies, early widespread microvascular alterations and fibrosis of the skin and internal organs (1-3). Microvascular damage, which cannot be compensated by an adequate and functional angiogenesis, clinically manifests as Raynaud's phenomenon, nailfold videocapillaroscopy (NVC) abnormalities, digital ulcers, pulmonary arterial hypertension and scleroderma renal crisis, while interstitial lung disease (ILD) represents the prominent fibrotic complication accounting for the high mortality among SSc patients (1, 2). Despite the overall insufficient angiogenic response, several studies reported that the proangiogenic mediator vascular endothelial growth factor-A (VEGF-A) is overexpressed in the skin and circulation of patients with SSc (4). Such an apparent discrepancy has been clarified by the discovery of the occurrence of an alternative splicing of the last exon (exon 8) of VEGF-A pre-mRNA, a process representing a key element in the switch from proangiogenic VEGF-$A_{165}$ to antiangiogenic VEGF-$A_{165}$b isoform (5, 6) and mediated by the regulatory splicing factor mRNA-binding protein serine/arginine protein 55 (*SRp55*, also known as SRSF6 or B52) (7). From a molecular point of view, VEGF-$A_{165}$b has the same affinity as VEGF-$A_{165}$ in binding VEGF receptor 2, but does not activate receptor phosphorylation or stimulate downstream intracellular signaling pathways, thus competitively inhibiting VEGF-$A_{165}$-mediated proangiogenic activity (6). Interestingly, overexpression of both VEGF-$A_{165}$b and *SRp55* has been implicated in SSc-related angiogenesis impairment and peripheral vascular damage (8, 9). Of note, previous studies reported the lack of sequence variations in the VEGF-A alternatively spliced region, while a single nucleotide polymorphism (SNP) in the *SRp55* gene (rs2235611) has been associated with the control of VEGF-A isoforms and susceptibility to angiogenic eye disease (10). Noteworthy, VEGF-$A_{165}$b has been reported to be also implicated in fibrosis, as it has been found to be significantly upregulated in lung tissue, isolated lung fibroblasts and plasma of patients with progressive idiopathic pulmonary fibrosis (IPF) (11). On these bases, the present case-control pilot study examined the possible implication of the *SRp55* rs2235611 SNP in the genetic predisposition to SSc susceptibility and clinical phenotype.

## Patients and methods

### *Study population and study design*

Based on a hypothesis-driven candidate gene approach, we performed a case-control pilot study which included a total of 872 white individuals from an Italian Caucasian population, comprising 414 SSc patients attending the outpatient clinic of the Division of Rheumatology (University of Florence, Italy) and 458 healthy controls matched by age, sex, ethnicity and geographical origin. At enrolment, a detailed clinical history was established for SSc patients by reviewing their medical records, with patients with overlap autoimmune and/or connective tissue diseases being excluded from the study. SSc patients were classified according to the 2013 ACR/EULAR SSc classification criteria (12) and assessed for limited and diffuse cutaneous subsets (lcSSc and dcSSc, respectively), anticentromere and anti-topoisomerase I antibodies (ACA and ATA, respectively), ILD defined as the presence of typical features on high-resolution CT scan of the chest, and NVC pattern. Demographic and clinical characteristics of SSc patients are represented in Table I. The study was approved by the local institutional review board, and written informed consent was obtained from all study subjects.

### *Genotyping*

Extraction of genomic DNA from the venous blood was performed using the FlexiGene DNA kit (Qiagen, Italy). The *SRp55* rs2235611 SNP was genotyped using TaqMan 5' allelic discrimination assay (predesigned assay with ID: C__27165073_10; Applied Biosystems, Foster City, California, USA). The PCR reaction was carried out according to instructions provided by the manufacturer. Post-PCR, the genotype

of each sample was automatically attributed by measuring the allele-specific fluorescence in a StepOnePlus real-time PCR System (Applied Biosystems). Representative allelic discrimination plot is shown in Fig. 1. The average genotype completeness for *SRp55* rs2235611 polymorphism was 98% for the SSc and the control samples. The accuracy was greater than 99%, according to duplicate genotyping of 10% of all samples.

*Statistics*

Statistical analyses were performed using SPSS software for Windows, v. 27.0 (SPSS Inc., Chicago, IL, USA). Categorical and quantitative variables were described as numbers and percentages and as the mean ± SD, respectively. We tested for deviation from Hardy-Weinberg equilibrium by standard chi-square test (1 degree of freedom) assessing the differences between observed genotype and expected genotype distributions based on control population allele frequencies. Allele frequency of the *SRp55* rs2235611 SNP was obtained by direct count. Genotype distribution and allele frequency of the *SRp55* rs2235611 SNP in SSc patients and controls were compared by chi-square analysis. The association between the *SRp55* rs2235611 SNP and SSc or clinical phenotypes (cutaneous subset, autoantibody status, presence/absence of ILD and NVC pattern) was assessed using a standard univariate logistic regression analysis. Odds ratios (ORs) with 95% confidence intervals (95% CIs) were determined. The level of significance for all tests was set to a type I error rate of $\alpha$=5% ($p$<0.05). Bonferroni correction was applied to all 'hypothesis-generating steps' when comparing the SSc subgroups and controls (seven phenotypic subsets). Values of $p$ after this adjustment for multiple testing are termed "$p_{adj}$".

## Results

No deviation from the expected population genotype proportions predicted by Hardy-Weinberg equilibrium was detected in patients with SSc or healthy controls at the *SRp55* rs2235611 polymorphic site. *SRp55* rs2235611 genotype distribution and allele frequency were similar in SSc and healthy controls, though a trend toward significance was observed for genotype distribution (genotypes 66.9% GG, 28.7% AG, 4.4% AA *vs*. 67.5% GG, 30.8% AG, 1.7% AA, respectively; chi-squared = 5.25; $p$=0.07) (Table II). The *SRp55* rs2235611 AA genotype significantly influenced the predisposition to SSc (OR 2.55, 95% CI 1.09–5.94, $p$=0.03), and to both lcSSc (OR 2.83, 95% CI 1.17–6.84, $p$=0.02) and dcSSc (OR 3.42, 95% CI 1.22–9.64, $p$=0.02) subtypes (Table II). No significant difference in genotype distribution and allele frequencies was observed between lcSSc and dcSSc subsets, as well as according to autoantibody status, between patients with ILD and those without ILD, and when comparing patients with early/active and those with late NVC pattern (data not shown). A trend toward an association between the AA genotype and ATA-positive SSc was found (OR 2.72, 95% CI 0.93–7.99, $p$=0.07) (Table II). The *SRp55* rs2235611 A minor allele and AA genotype showed a significant risk association with susceptibility to SSc-related ILD (A allele: OR 1.39, 95% CI 1.00–1.93, $p$=0.046; AA

**Table I.** Demographic and clinical characteristics of SSc patients.

| Characteristics | SSc population (n=414) |
|---|---|
| Female, n (%) | 348 (84.0) |
| Mean age (years ± SD) | 56.8 ± 16.2 |
| Limited cutaneous SSc, n (%) | 296 (71.5) |
| Diffuse cutaneous SSc, n (%) | 118 (28.5) |
| ACA+ patients, n (%) | 174 (42.0) |
| ATA+ patients, n (%) | 130 (31.4) |
| ILD, n (%) | 143 (34.5) |
| Early/active NVC, n (%) | 276 (66.7) |
| Late NVC, n (%) | 138 (33.3) |

ACA: anticentromere antibodies; ATA: anti-topoisomerase I antibodies; ILD: interstitial lung disease; NVC: nailfold videocapillaroscopy; SD: standard deviation; SSc: systemic sclerosis.

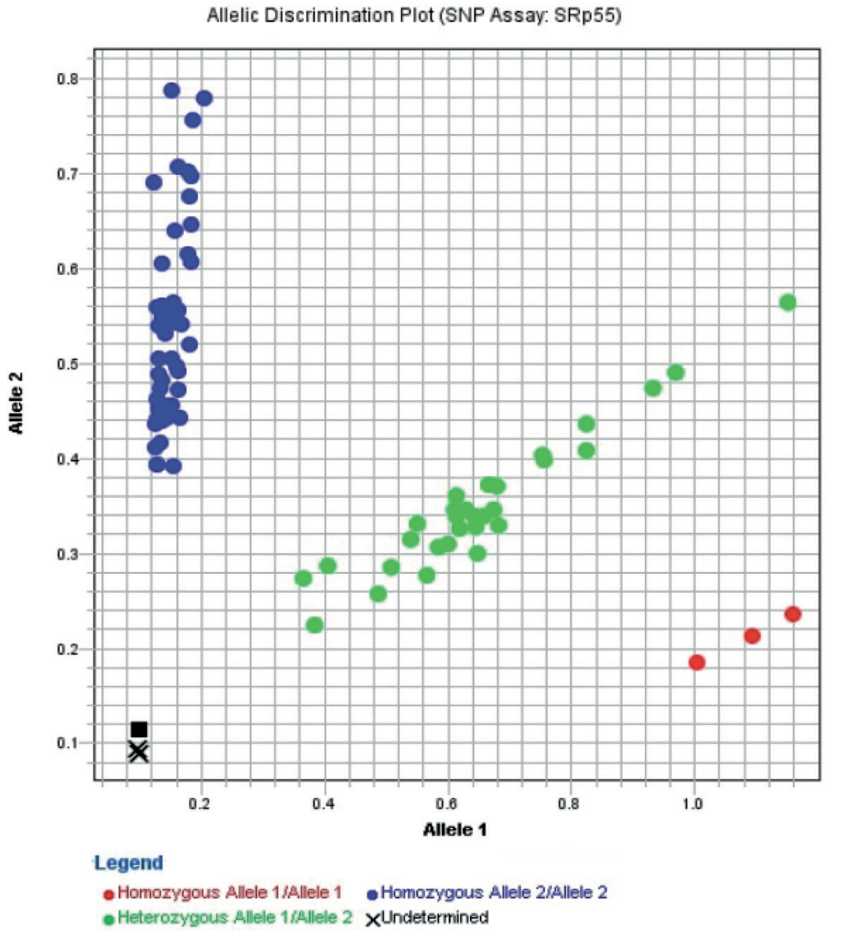


**Fig. 1.** Representative *SRp55* rs2235611 allelic discrimination plot.

**Table II.** Genotype and minor allele frequencies of the *SRp55* rs2235611 SNP in patients with SSc and controls.

| Subgroup | Genotype, n (%) | | | MAF (%) | | *p*-value | $p_{adj}$ value* | OR (95% CI) |
|---|---|---|---|---|---|---|---|---|
| | GG | AG | AA | | | | | |
| Controls (n=458) | 309 (67.5) | 141 (30.8) | 8 (1.7) | 17.13 | | NA | NA | NA |
| SSc (n=414) | 277 (66.9) | 119 (28.7) | 18 (4.4) | 18.72 | A | 0.38 | | 1.11 (0.87 to 1.42) |
| | | | | | AA | **0.03** | | 2.55 (1.09 to 5.94) |
| lcSSc (n=292) | 192 (65.7) | 86 (29.4) | 14 (4.9) | 19.52 | A | 0.24 | | 1.17 (0.89 to 1.53) |
| | | | | | AA | **0.02** | | 2.83 (1.17 to 6.84) |
| dcSSc (n=122) | 79 (64.7) | 36 (29.5) | 7 (5.8) | 20.49 | A | 0.22 | | 1.25 (0.87 to 1.78) |
| | | | | | AA | **0.02** | | 3.42 (1.22 to 9.64) |
| ACA+ (n=174) | 135 (77.6) | 53 (30.5) | 8 (8.1) | 19.83 | A | 0.84 | | 1.03 (0.76 to 1.41) |
| | | | | | AA | 0.08 | | 2.39 (0.88 to 6.47) |
| ATA+ (n=130) | 82 (63.1) | 42 (32.3) | 6 (4.6) | 20.77 | A | 0.18 | | 1.27 (0.89 to 1.79) |
| | | | | | AA | 0.07 | | 2.72 (0.93 to 7.99) |
| ILD (n=143) | 88 (61.5) | 46 (32.2) | 9 (6.3) | 22.38 | A | **0.046** | | 1.39 (1.00 to 1.93) |
| | | | | | AA | **0.007** | **0.049** | 3.78 (1.43 to 9.98) |
| Early/active NVC (n=276) | 187 (67.7) | 80 (29.0) | 9 (3.3) | 17.75 | A | 0.94 | | 1.01 (0.74 to 1.39) |
| | | | | | AA | 0.19 | | 1.90 (0.72 to 4.97) |
| Late NVC (n=138) | 90 (65.2) | 39 (28.3) | 9 (6.5) | 20.65 | A | 0.62 | | 0.90 (0.60 to 1.35) |
| | | | | | AA | **0.006** | **0.042** | 3.92 (1.48 to 10.37) |

Controls have been used as reference for all comparisons. Bold indicates statistically significant *p*-values at α=5%.
**p*<0.05 adjusted after Bonferroni correction for multiple comparisons.
ACA: anticentromere antibodies; ATA: anti-topoisomerase I antibodies; CI: confidence interval; dcSSc: diffuse cutaneous systemic sclerosis; ILD: interstitial lung disease; lcSSc: limited cutaneous systemic sclerosis; MAF: minor allele frequency; NA: not applicable; NVC: nailfold videocapillaroscopy; OR: odds ratio; SNP: single nucleotide polymorphism; SSc: systemic sclerosis.

genotype: OR 3.78, 95% CI 1.43–9.98, *p*=0.007) (Table II). Moreover, the AA genotype was found to be significantly associated with the presence of a late NVC pattern (OR 3.92, 95% CI 1.48–10.37, *p*=0.006) (Table II).

After Bonferroni correction for multiple comparisons, the risk association of the *SRp55* rs2235611 AA genotype with SSc-related ILD and late NVC pattern remained significant ($p_{adj}$=0.049 and $p_{adj}$=0.042, respectively) (Table II).

## Discussion

In this case-control pilot study, we analysed for the first time the possible contribution of the *SRp55* gene in modulating both the predisposition to and the clinical phenotype of SSc, a complex connective tissue disorder characterised by microvascular abnormalities, disturbed angiogenesis and fibrosis of the skin and internal organs (1-3). In particular, we selected as genetic marker the rs2235611 SNP, that has been previously implicated in angiogenic eye disease (10). Our study shows that the *SRp55* rs2235611 AA genotype not only significantly influences the susceptibility to SSc, but specifically associates with the presence of SSc-related ILD and late NVC pattern.

Increasing evidence suggests that SSc is a complex polygenic disorder whose pathogenesis and evolution appear to be modulated by the interaction between multiple genetic and environmental components and in which different SNPs may play a relevant role in disease progression and clinical phenotype, or both (13-15). Our data providing evidence for an association of the *SRp55* rs2235611 gene variant with SSc-related lung fibrosis are concordant with a previous study demonstrating the role of *SRp55* in pleural fibrosis (16). Indeed, it has been demonstrated that this splicing factor mediates collagen synthesis in human primary pleural mesothelial cells and that the *in vivo* knock-out prevents pleural fibrosis in the bleomycin-induced mouse model (16). *SRp55* is known to regulate the alternative splicing of VEGF-A pre-mRNA, leading to a higher production of the antiangiogenic VEGF-$A_{165}$b isoform (7). Interestingly, a previous study from our group demonstrated an increased expression of both *SRp55* and VEGF-$A_{165}$b in the skin of SSc patients (8), and a significant overexpression of such an antiangiogenic isoform has been also detected in the lungs of patients with IPF (11). On these bases, although the expression of *SRp55* has not yet been evaluated in SSc lung tissue, it is tempting to speculate that this splicing factor might be overexpressed at this anatomic site as well, possibly contributing to the development of pulmonary fibrosis in such patients. In addition, the association of the *SRp55* rs2235611 gene variant with the late NVC pattern, which is characterised by substantial peripheral capillary loss and lack of angiogenesis, is consistent with our previous findings reporting that increased plasma levels of VEGF-$A_{165}$b are associated with the severity of nailfold capillary impairment (9). However, we must consider that the associations found in our small pilot study have to be confirmed in additional larger independent cohorts of SSc patients. Moreover, the potential functional role of *SRp55* gene variations in influencing SSc-related ILD and peripheral microvasculopathy remains to be investigated. Indeed, although the herein analysed SNP is synonymous

(*i.e.* not causing a change in the amino acid), we cannot exclude the possibility that a linkage disequilibrium between *SRp55* rs2235611 and other nonsynonymous/functional SNPs spanning the *SRp55* gene might affect SSc susceptibility and its clinical phenotype. In this context, we should also take into account that increasing evidence suggests that even synonymous mutations may alter the structure, function, and expression level of proteins through the regulation of mRNA splicing, stability, and structure as well as protein folding (17). Finally, it is worth considering that *SRp55* is not specific for alternative splicing of VEGF-A pre-mRNA (18) and, therefore, it could be of interest to also explore whether this splicing factor and its gene variations may affect the expression of other factors involved in the pathogenesis of SSc. Since increasing evidence suggests that deciphering the genetic bases of SSc may be crucial for a better disease classification and the development of novel therapies (19), further in-depth studies on the *SRp55* gene locus will hopefully contribute to extend our knowledge of the genetic predisposition to major SSc-related clinical manifestations such as pulmonary fibrosis and peripheral microvascular impairment.

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