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Detection of anti-IFI16 antibodies by ELISA: clinical and serological associations in systemic sclerosis

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BASIC SCIENCE

Abstract

Objectives. To validate the clinical significance of anti-IFI16 autoantibodies in SSc and assess their associations with serological markers of SSc.

Methods. A semi-quantitative ELISA was used to detect anti-IFI16 autoantibodies in the sera of 344 SSc patients from seven Italian hospitals and 144 healthy controls. SSc-associated autoantibodies [anti-RNA polymerase III (anti-RNAP III) antibodies, anti-centromere, anti-topo I] and IF patterns were evaluated using commercial assays. Statistical analyses were performed to test clinical and serological associations.

Results. The results of this study confirm a significant prevalence (29%) of anti-IFI16 antibodies in the SSc population ($n = 344$). Anti-IFI16 antibodies were also detected in 30% of the SSc patients who tested negative for both ACAs and anti-topo I (anti-Scl70) antibodies. In this subgroup of patients, anti-IFI16 antibodies were significantly associated with the limited cutaneous form of SSc with a sensitivity of 40% and a specificity of 81%. Moreover, analysis of the distribution of anti-RNAP III antibodies *vs* anti-IFI16 in the same SSc population showed that they were mutually exclusive. IIF revealed no association between anti-IFI16 and fluoroscopic patterns, due to a lack of IFI16 autoantigen in HEp-2 cells. Anti-IFI16 antibody levels were also significantly associated with heart involvement.

Conclusions. Anti-IFI16 autoantibodies are frequently detected in SSc, displaying clinical and laboratory associations, and being particularly useful for diagnosis and disease classification in patients who are negative for other SSc serological markers.

Key words: Systemic sclerosis, IFI16, Autoantibodies, Diagnosis, Interferon.

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Introduction

SSc is an autoimmune multisystem connective tissue disorder characterized by inflammatory and fibrotic processes that result in changes to skin, blood vessels and internal organs including lung, gastrointestinal tract, kidney and heart. The aetiology and pathogenesis of SSc are unknown; however, immunological abnormalities, fibroblast activation, chronic inflammation and vascular damage are considered to be the main elements of the disease [1]. The two widely recognized subsets of SSc are lcSSc and dcSSc. These two forms of SSc present divergent patterns of internal organ involvement, clinical course and autoantibody profiles, as defined by the LeRoy criteria [2]. In dcSSc, the condition may develop within weeks to involve the internal viscera, while patients with the lcSSc usually remain stable for years, although they may develop late visceral complications [3]. SSc, like many other systemic autoimmune disorders, is also characterized by the occurrence of ANAs. ACAs, anti-DNA topo I (anti-Scl70) and anti-RNA polymerase (RNAP I–III) autoantibodies are the most common and are specific to SSc. Anti-fibrillarin antibody (AFA), also called anti-U3-RNP, and anti-Th/To are also regarded as being specific to SSc, even if they are only found in a low proportion of patients. Antibodies to SSA/Ro, SSB/La or anti-U1-RNP may also be present, but have little specificity to scleroderma [4]. Associations between anti-Scl70, ACA and anti-RNAP III antibodies and organ involvement have been reported. Patients with anti-Scl70 antibodies are prone to develop pulmonary fibrosis, while ACAs are associated with pulmonary arterial hypertension. Anti-RNAP III antibodies have been associated with an increased risk of renal crisis [3], although such a correlation was not confirmed in a recent study of Italian SSc patients [5].

The family of human IFN-inducible genes designated HIN-200 encodes evolutionarily related nuclear phosphoproteins [IFI16, myeloid cell nuclear differentiation antigen (MNDA), absent in melanoma 2 (AIM2), pyrin and HIN domain family member 1 (IFIX)]. Physiological IFI16 expression was found in cells of the immune system, in endothelial cells and in stratified squamous epithelia, such as skin [6]. Several cellular activities have been assigned to IFI16, including roles in inflammatory and apoptotic processes [7]. In line with these observations, overexpression of IFI16 in primary human umbilical vein endothelial cells efficiently suppresses tube morphogenesis *in vitro* and increases expression of pro-inflammatory molecules [8, 9]. Immunohistochemical analyses of cutaneous lesions obtained from patients of SSc and SLE showed increased IFI16 expression in both epithelial and inflammatory cells [10]. The vascular system and the skin represent the target organs of several autoimmune diseases, including scleroderma, indicating that IFI16 may be involved in autoimmune processes. According to this hypothesis, anti-IFI16 antibodies have been detected in SLE by immunoblotting [11] and in primary/secondary SS by serological analysis of antigens by recombinant cDNA expression cloning [12]. By means of a semi-quantitative ELISA, we have previously reported that anti-IFI16 autoantibodies are present in a significant fraction of SSc patients [10]. The results of this monocentric study also showed that anti-IFI16 is useful for differentiating lcSSc and dcSSc. It was therefore of significant interest to corroborate these results in a wider cohort of SSc patients.

The objective of the present multicentric study, performed in collaboration with the organization Forum Interdisciplinare per la Ricerca nelle Malattie Autoimmuni (FIRMA), was to validate the clinical significance of anti-IFI16 autoantibodies. The analysis, performed in 344 patients recruited from seven different hospitals, confirmed a significant prevalence of anti-IFI16 in the SSc population. Moreover, we observed that anti-IFI16 are the only autoantibodies detected in a proportion of SSc patients. Even if the association of anti-IFI16 with the limited cutaneous subset of SSc is limited to a trend in the present population, new and interesting clinical associations were nonetheless identified.

Patients and methods

Patients and controls

Seven groups of SSc patients were included in this study: 6 patients from University Campus Biomedico (Rome, Italy); 6 patients from Policlinico di Monteluce (Perugia, Italy); 23 patients from Ospedale Civile (Legnano, Milano, Italy); 75 patients from Policlinico Le Scotte (Siena, Italy); 75 patients from University Sapienza (Rome, Italy); 24 patients from Azienda Ospedialiera S. Camillo-Forlanini (Rome, Italy); and 135 patients from Azienda Ospedaliera Spedali Civili (Brescia, Italy). All patients fulfilled the ACR classification criteria for definite SSc [13]. Sera from 144 sex- and age-matched healthy subjects were collected from blood banks and composed the control group. Informed consent was obtained from all participants according to the Declaration of Helsinki. Approval was obtained from local ethics committees [Ethics Committee of Istituto Auxologico Italiano (2010_07_22_09) and Ethics Committee of Spedali Civili of Brescia (152, 23 February 2009)].

Clinical and serological parameters

The clinical, instrumental and laboratory data reported in this study were obtained at the time the blood samples were drawn. All patients with SSc [(302 women and 42 men, mean age 64 years (range 12–83 years)] were classified as having lcSSc or dcSSc according to LeRoy and Medsger [2]. Patients' data were collected by using the Minimal Essential Data Set (MEDS) from EULAR Scleroderma Trials and Research group (EUSTAR) [14]. In brief, the clinical features of the patients were defined as follows: pulmonary fibrosis = bibasilar pulmonary fibrosis on chest radiography; pulmonary hypertension = clinical evidence of pulmonary hypertension and increased mean pulmonary arterial pressure (>35 mmHg), indirectly assessed by echocardiography, in the absence of severe pulmonary interstitial fibrosis;

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gastrointestinal = oesophageal hypomotility shown by barium radiography; heart = pericarditis, congestive heart failure, arrhythmias requiring treatment, conduction system abnormalities or diastolic dysfunction. ANAs and ACAs were detected by IF using HEp-2 cells as substrate. Anti-ENA antibodies, including anti-topo I were evaluated by CIE and/or EIAs.

Determination of antibody titres towards human recombinant IFI16 by ELISA

Polystyrene micro-well plates (Nunc-Immuno MaxiSorp; Nunc, Roskilde, Denmark) were coated with a solution of recombinant IFI16 in PBS. After blocking, sera were added in duplicate. After washing, horseradish peroxidase-conjugated rabbit anti-human IgG (Dako Cytomation, Carpinteria, CA, USA) was added. Following the addition of the substrate (TMB; KPL, Gaithersburg, MD, USA), absorbance was measured at 450 nm, using a microplate reader (TECAN, Männedorf, Switzerland). The assay was performed using the Freedom Evolyzer instrument (TECAN). The background reactivity of the reference mixture was subtracted to calculate the results. A standard curve was constructed by serially diluting anti-IFI16-positive patient sera.

Determination of antibody titres towards RNAP III by ELISA

Anti-RNAP was detected using a commercially available ELISA kit (Anti-RNA Polymerase III ELISA Kit; MBL International, Woburn, MA, USA) [15]. The ELISA was performed in accordance with the manufacturer's instructions and using the recommended cut-off of 28 U/ml.

IIF assay

The detection and titration of anti-nuclear autoantibodies in human sera was performed using an ANA Kit (HEp-2 cells; Astra, Milan, Italy) and according to the manufacturer's protocol. To analyse the distribution of IFI16 in HEp-2 cells, anti-IFI16 antibodies {C-term polyclonal (working dilution 1 : 800) [6]} were applied to HEp-2 cells on slides obtained from four different manufacturers (Astra, The Binding Site, Birmingham, UK; BioRad, Segrate, Italy; Inova, San Diego, CA, USA) and incubated for 30 min at room temperature (RT). For the determination of IFI16 in normal human epithelial keratinocytes (NHEKs), cells were grown on glass slides and then fixed in 4% paraformaldehyde for 2 min at RT. Cells were permeabilized with 0.2% Triton-X100 in PBS for 20 min at 4°C. After blocking, coverslips were incubated for 1 h with anti-IFI16 antibodies {C-term polyclonal (working dilution 1 : 800), [6]}. For antibody detection, cells were then incubated with FITC-labelled secondary antibody [goat anti-rabbit IgG, Alexa Fluor 488 (working dilution 1 : 200); Molecular Probes, OR, USA] for 1 h in the dark at RT. IF was observed using a fluorescence microscope (DMI 6000 B; Leica, Milan, Italy).

Statistical analysis

Statistical analysis was performed using Prism 5 software (GraphPad, La Jolla, CA, USA). Positivity cut-off values for anti-IFI16 antibodies were calculated as the 95th percentile for the control population. The Mann–Whitney test or Fisher's exact test was used to measure associations. Correlations were evaluated by Spearman's test. $P \leqslant 0.05$ were considered statistically significant.

Results

The study cohort was composed of 344 patients with a confirmed diagnosis of SSc recruited from seven Italian hospitals. Sera were processed by ELISA to evaluate the levels of anti-IFI16 autoantibodies. In line with our previous observations [8], significantly higher anti-IFI16 titres were observed in SSc patients compared with age- and sex-matched healthy controls (median levels: 42.5 *vs* 23.5 U/ml; $P < 0.0001$; Fig. 1). With the cut-off level set to the 95th percentile of the control population (89 U/ml), 29% of the patients tested positive for anti-IFI16 autoantibodies. The distribution of anti-IFI16 titres showed no significant difference between the different centres (data not shown).

Clinical and laboratory data were available for the patients, thus univariate analyses were performed in order to investigate whether associations existed between the anti-IFI16 autoantibody titres and other clinical or serological characteristics of the patients. Consistent with our previous results [10], anti-IFI16 titres did not associate

Fig. 1 IgG titres by ELISA assay against human recombinant IFI16 in patients with SSc ($n = 344$) and healthy controls (CTRL, $n = 144$). Each dot represents the anti-IFI16 level from a single sample (expressed in AU/ml on a $\log_2$ scale). The horizontal bars represent the median values. Values over the dotted line indicate the percentage of subjects with IgG titres above the cut-off value (89 U/ml), calculated as the 95th percentile of the control population. Statistical significance: *$P < 0.0001$ *vs* controls (Mann–Whitney test).

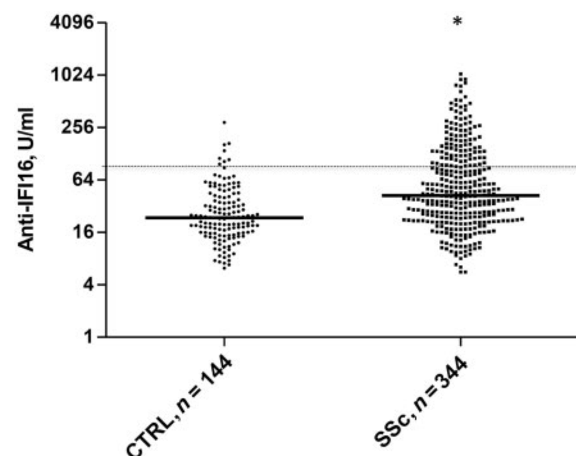

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TABLE 1 Clinical and laboratory features of SSc patients positive or negative to IgG anti-IFI16

	IgG anti-IFI16 antibody (+), *n* = 100	IgG anti-IFI16 antibody (−), *n* = 244	*P*-value
Sex, males/females, *n*	13/84	26/214	0.5728
Age, mean (S.D.), years	61 (13)	57 (13)	**0.0018**
Disease pattern, lcSSc, %	68	57	0.0633
Capillaroscopy, pathological, %	80	85	0.3247
Organ involvement			
Lungs			
Pulmonary fibrosis, %	52	45	0.2810
DL_{CO}/VA or DL_{CO}/Hb <75%, %	44	46	0.9006
Pulmonary hypertension, %	25	23	0.6711
Heart, %	47	34	**0.0264**
Muscles, %	7	7	1.000
Gastrointestinal, %	64	65	0.9002
Laboratory findings			
Positive for anti-topo I antibody, %	27	26	1.0000
Positive for ACA, %	38	41	0.7121

Bold letters indicate $P \leqslant 0.05$. DL_{CO}/VA: diffusion lung capacity for carbon monoxide/alveolar volume corrected; DL_{CO}/Hb: diffusion lung capacity for carbon monoxide/haemoglobin corrected.

with sex, pathological capillaroscopic features or positivity to other autoantigens (Table 1). The mean age of patients who tested positive for anti-IFI16 was found to be slightly higher than that of patients negative for anti-IFI16 [mean age (S.D.): 61 (13) *vs* 57 (13) years, respectively]. The association between anti-IFI16 positivity and the limited cutaneous subset of the disease nearly reached a statistically significant level ($P = 0.0633$). Analysis of internal organ involvement in the SSc population revealed a significant and positive association between anti-IFI16 and cardiac complications ($P = 0.0264$). When patients were divided into two groups according to the presence or absence of cardiac dysfunction, we found significantly higher anti-IFI16 titres in patients displaying heart involvement (see supplementary figure, available as supplementary data at *Rheumatology* Online; $P = 0.0307$). Nevertheless, the limited median difference between the two groups (52.58 *vs* 38.16 U/ml) did not support the use of a quantitative analysis of anti-IFI16 antibodies to evaluate an increased risk of cardiac dysfunction.

In accordance with the prevalence previously reported for the Caucasian population [16], 66% of the SSc patients were positive for anti-topo I (anti-Scl70) and/or ACAs in the study population (data not shown). In line with the results of our earlier monocentric study, the presence of anti-IFI16 autoantibodies was not associated with the presence of either anti-Scl70 or ACA autoantibodies, but there was a partial overlap with both markers (Table 1); however, anti-IFI16 was consistently present in patients negative for these two markers [anti-Scl70 and ACA double-negative SSc patients (dnSSc)]. Indeed, 30% of the dnSSc patients displayed anti-IFI16 levels higher than the cut-off value (Fig. 2A), thus confirming our previous finding indicating that anti-IFI16 can be used as a supplementary marker of SSc. In addition, when we compared the occurrence of lcSSc *vs* dcSSc in the dnSSc subgroup, anti-IFI16 positivity was significantly associated with lcSSc ($P = 0.0319$), with a sensitivity of 40% and a specificity of 81% for discrimination of the cutaneous subset (Fig. 2B). No other association was found in the dnSSc patients (data not shown).

Anti-RNAP III autoantibodies are known to segregate with ACA and Scl70 negativity [17]. It was therefore of particular interest to analyse the distribution of anti-IFI16 and anti-RNAP III in our cohort of dnSSc patients. In order to do so, the presence of anti-RNAP III was analysed in 88 consecutive dnSSc patients using a commercial assay. In line with previous reports [17, 18], the presence of anti-RNAP III was found to be significantly associated with dcSSc ($P = 0.0045$, Fisher's exact test; data not shown), while no correlation was found between anti-IFI16 and anti-RNAP III titres. As depicted in Fig. 3, nearly all patients displaying high levels of anti-IFI16 were negative for anti-RNAP III and vice versa. Indeed, only three patients out of 88 were found positive for both markers, indicating that anti-IFI16 and anti-RNAP III autoantibodies are mutually exclusive. Taken together, these results justify the combination of the four autoantibodies tested (ACA, anti-Scl70, anti-RNP III and anti-IFI16), which allows a degree of sensitivity of 81% for the identification of SSc patients to be reached (Fig. 4).

To analyse whether anti-IFI16 autoantibodies could be associated with a characteristic fluoroscopic pattern, sera from 40 anti-IFI16-positive SSc patients were tested by IIF and detailed image analysis was performed. Eight different anti-nuclear patterns were found (see supplementary table, available as supplementary data at *Rheumatology* Online), but none of them displayed any predominance. The distribution of the principal ANAs in the anti-IFI16-positive subgroup reflected that of the overall

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FIG. 2 Analysis of the anti-IFI16 distribution in patients with SSc double negative for anti-Scl70 and ACA (dnSSc). (**A**) IgG titres by ELISA assay against human recombinant IFI16 in dnSSc ($n = 114$) and healthy controls (CTRL, $n = 144$). Each dot represents the anti-IFI16 level from a single sample (expressed in AU/ml on a $\log_2$ scale). The horizontal bars represent the median values. Values over the dotted line indicate the percentage of subjects with IgG titres above the cut-off value (89 U/ml) calculated as the 95th percentile of the control population. Statistical significance: $^*P < 0.0001$ *vs* controls (Mann–Whitney test). (**B**) Contingency analysis of the usefulness of anti-IFI16 for the differential diagnosis of lcSSc from dcSSc. The white columns represent the number of patients positive to anti-IFI16 with IgG titres above the cut-off value and the black columns represent the number of patients negative to anti-IFI16. Statistical significance: $P = 0.0342$ (Fisher's exact test).

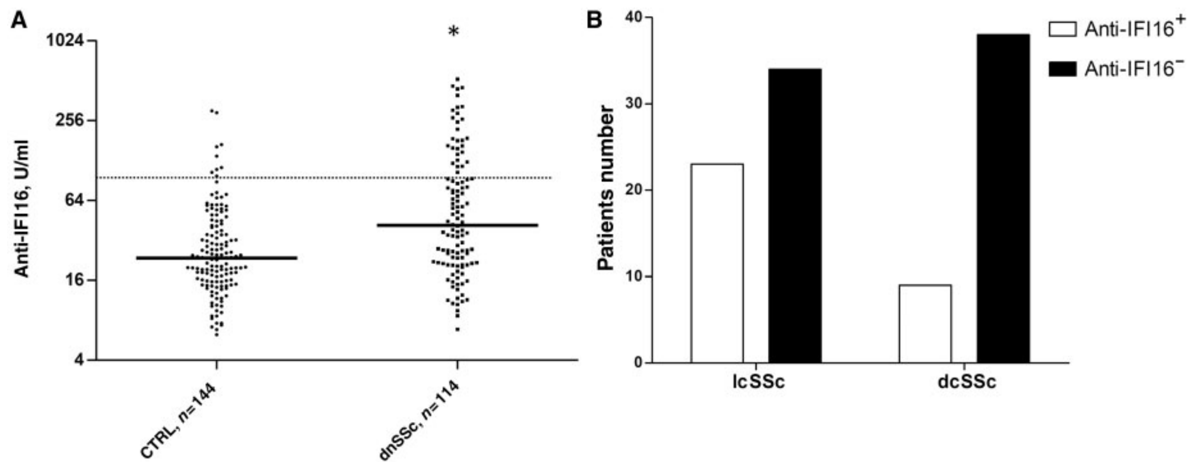
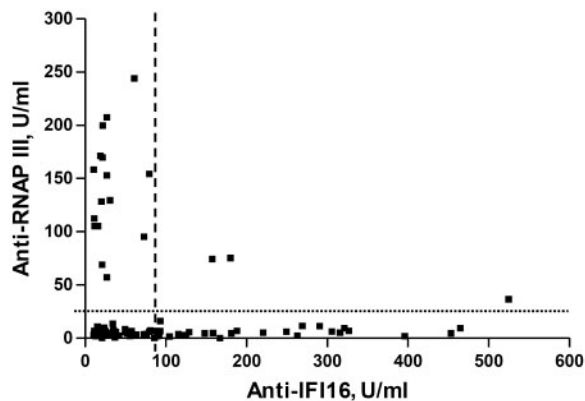


FIG. 3 Distribution of IgG titres of anti-IFI16 (*x*-axis) and anti-RNAP III (*y*-axis) in patients with SSc testing double negative for Scl70 and ACA (dnSSc, $n = 88$). Dashed and dotted lines represent the cut-off values for anti-IFI16 (89 U/ml) and anti-RNAP III (28 U/ml), respectively.



SSc population (speckled = 32%; homogeneous = 16%; data not shown). Surprisingly, three of the sera tested were negative to IIF analysis (see supplementary table, available as supplementary data at *Rheumatology* Online) despite the presence of high anti-IFI16 titres. These findings led us to investigate whether the IFI16 protein was present in HEp-2 cells by using rabbit polyclonal anti-IFI16 antibodies. A very weak immunofluorescent signal was detected in HEp-2 cells, while a strong homogeneous nuclear signal was observed in normal human keratinocytes (Fig. 5). Importantly, similar results were obtained using kits from four different manufacturers of HEp-2 substrate (data not shown). Collectively, our data indicate that HEp-2 cells express very low, barely detectable, amounts of IFI16, which could account for the lack of an association between the presence of anti-IFI16 autoantibodies and a specific fluoroscopic pattern.

Discussion

The presence of anti-IFI16 autoantibodies in SSc patients was first described in 2006, as result of a monocentric study performed by our group [10]. The aims of the present multicentric study were to confirm the prevalence of anti-IFI16 autoantibodies in a larger cohort of patients, and to better characterize their associations with clinical and laboratory findings. We observed a higher prevalence of anti-IFI16 in SSc compared with our previous findings (29 *vs* 21%), which was possibly due to a better definition of the cut-off level as a consequence of the larger healthy control group analysed in this study. The anti-IFI16-positive patients were significantly older than the anti-IFI16-negative patients, although the mean ages differed by only 4 years. We could not verify the hypothesis that the observed age difference was due to a longer disease duration since such data were lacking for a significant proportion of the patients. It is worth noting that a significant and positive association was found between anti-IFI16 antibodies and heart involvement. Although

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Fig. 4 Sensitivity analysis of the four laboratory markers of SSc tested. The grey segments represent the increase in sensitivity obtained when using one or more additional marker(s) to the gold standard ACA and anti-Scl70 detection.

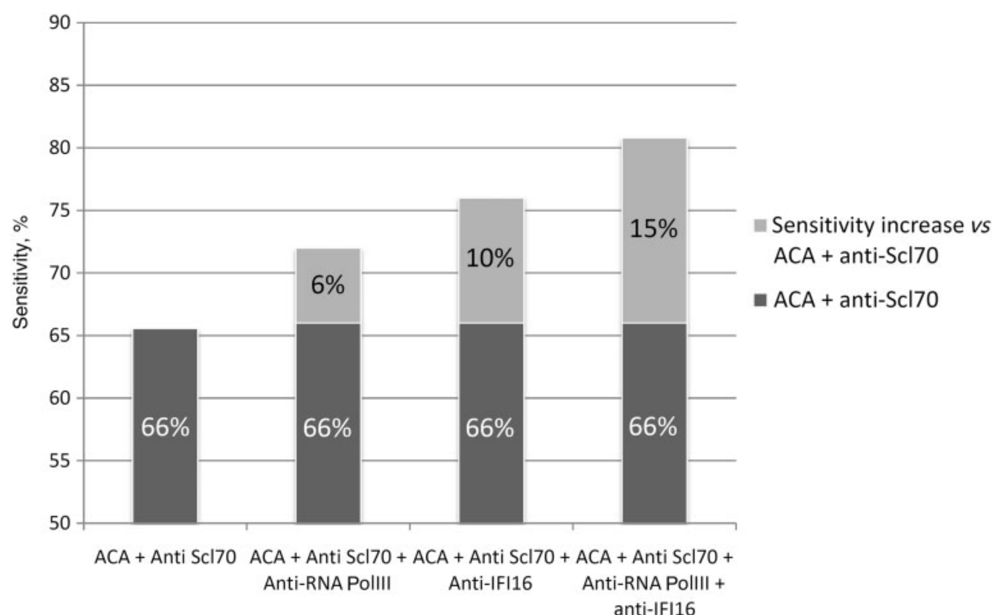
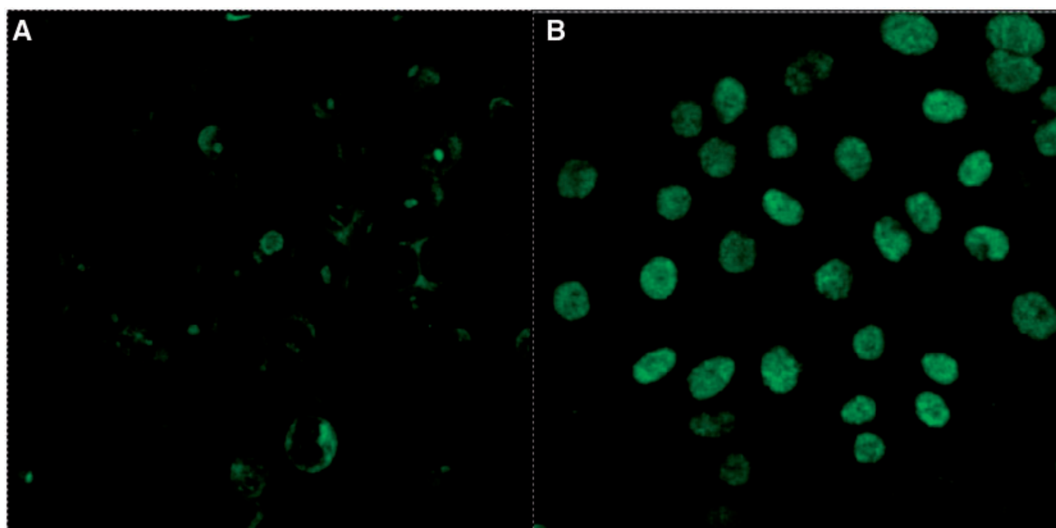


Fig. 5 IF analysis of IFI16 expression in HEp-2 cells (**A**) and NHEKs (**B**). Cells were incubated with anti-IFI16 rabbit polyclonal antibodies. Distribution of IFI16 autoantigen was revealed by FITC-conjugated anti-rabbit antibodies and fluorescent signals acquired by fluorescence microscopy.



the prognostic value of the anti-IFI16 autoantibodies needs to be verified by longitudinal studies, their potential use for anticipating the development of cardiac symptoms (among the leading causes of morbidity and mortality in SSc) is of great interest as such manifestations can silently progress undetected and result in a poor disease prognosis [19, 20].

In agreement with our previous observations [10], the distribution of anti-IFI16 positivity was partially overlapping with that of ACA and anti-Scl70. However, 30% of patients testing negative for the two main serological markers of SSc (anti-Scl70 and ACA) were positive for anti-IFI16 autoantibodies. Moreover, here we report that anti-IFI16 antibodies are mutually exclusive with anti-RNAP III antibodies, which have been described in dnSSc patients and are known to associate with dcSSc. This result is in line with the observation that anti-IFI16 significantly associate with lcSSc in the double-negative patients, confirming that they segregate with a different population with respect to anti-RNAP III. Several other

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minor autoantibodies have also been described in SSc patients [21], among which are scleroderma-specific autoantibodies such as AFA and anti-Th/To, but no such data were available for our study cohort. Further studies analysing the distribution of IFI16 with respect to these other SSc autoantibodies (in particular, anti-Th/To, which are known to segregate with lcSSc [22]) will allow a complete picture of the various SSc autoantibody profiles to be obtained.

IIF on HEp-2 cell substrate is often the first laboratory step in the diagnostic flowchart for SSc. Data relative to the presence of the two principal fluoroscopic patterns (homogeneous and speckled) were available for all patients, but no associations with anti-IFI16 positivity were found (data not shown). We then verified whether anti-IFI16 autoantibodies could be associated with less common fluoroscopic patterns by re-testing the sera of 40 anti-IFI16-positive SSc patients. The data analysis revealed no associations between anti-IFI16 and IIF patterns. Moreover, the lack of expression of IFI16 in HEp-2 cells clearly indicates that analysis of the IIF pattern is not able to predict whether anti-IFI16 autoantibodies are present or not, and also suggests that IIF-negative SSc patients should also be tested for the presence of anti-IFI16. Although the subcellular localization of the IFI16 autoantigen is well recognized as nuclear [6, 23], anti-IFI16 cannot be classified as a classical ANA, because it lacks HEp-2 staining, the gold standard for identifying ANAs.

In this study, we have assessed the diagnostic performance of an ELISA method in a large series of sera collected from seven medical centres situated in different areas of Italy. Our results indicate that anti-IFI16 antibodies are the third most prevalent autoantibodies found in SSc, even if it should be taken into account that they are not restricted to this disease. The evaluation of their presence can be useful in the disease classification of SSc, particularly in patients negative for other serological markers. Since ELISA can be performed quickly and easily, and is particularly suitable for screening large numbers of sera, this assay may represent a suitable test for detecting anti-IFI16 autoantibodies in clinical laboratories.

Rheumatology key messages

- Anti-IFI16 autoantibodies are present in a significant proportion of SSc patients (29%).
- Anti-IFI16 and anti-RNAP III autoantibodies are mutually exclusive.
- Anti-IFI16 autoantibodies are helpful for SSc classification, particularly in patients negative for other serological markers.

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Supplementary data

Supplementary data are available at *Rheumatology* Online.

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