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Review

Diagnostic accuracy and predictive value of extended autoantibody profile in systemic sclerosis

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ABSTRACT

Systemic sclerosis (SSc) is a heterogeneous autoimmune disorder characterized by microvascular injury, fibrosis of the skin and other organs, and presence of antinuclear autoantibodies (ANA) with a prevalence varying from 80 to 98%. The ANA classically detected in SSc include anti-centromere (ACA) and anti-topoisomerase I (ATA), which are positive in 50–60% of the patients. Even if other autoantibodies, such as anti-fibrillarin (AFA), anti-RNA polymerase III (RNAP III), anti-PMScI, anti-Th/To, and anti-hUF/NOR-90, are almost specific for SSc, until recently they were not routinely looked for, since the techniques for their identification were not suitable for routine use. In recent years, the advances in the knowledge of the biochemistry and of the immunoreactive sites of the autoantigens led to the development of new immunoassays using recombinant proteins as autoantigens. We evaluated a new multiplex line immunoblot assay (LIA) for the simultaneous detection of 13 different SSc-associated autoantibodies, in a cohort of 210 SSc Italian patients. The sensitivity and the specificity of this assay were as follows: 30.5% and 97.3% for ACA (anti-CENP-B), 29.5% and 96% for ACA (anti-CENP-A), 20% and 99.3% for ATA, 5.7% and 99.3% for anti-RNAP III (RP-155), 5.2% and 100% for anti-RNP III (RP-11), 6.7% and 98% for anti-PMScI (PMScI-100), 10.9% and 93.3% for anti-PMScI (PMScI-75), 3.3% and 98.7% for anti-Th/To, 0.48% and 100% for AFA, 4.8% and 96.7% for anti-hUF/NOR-90, 4.7% and 96% for anti-Ku, 0.95% and 100% for anti-Platelet-Derived Growth Factor Receptor, and 18.1% and 50% for anti-Ro-52, respectively. These results, which are similar to those obtained in other studies using traditional techniques, show that the LIA assay can be considered a more rapid and a more practical method than immunoprecipitation assays for studying SSc-related antibodies in the diagnostic work-up of SSc patients.

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Contents

1. Introduction	0
2. Material and methods	0
2.1. Patients	0
2.2. Autoantibody detection	0
2.3. Statistical analysis	0
3. Results	0
4. Discussion	0
4.1. Anti-RNAP III antibodies	0
4.2. Anti-PMScI antibodies	0
4.3. Anti-fibrillarin antibodies	0
4.4. Anti-Th/To antibodies	0
4.5. Anti-Ku antibodies	0

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60	4.6. Anti-hUBF/NOR-90 antibodies	0
61	4.7. Anti-PDGF antibodies	0
62	Take-home messages	0
63	Acknowledgments	0
64	References	0

1. Introduction

Systemic sclerosis (SSc) is an autoimmune connective tissue disease characterized by microvascular injury and immunological abnormalities leading to fibrosis of the skin and other organs [1–7]. SSc patients have autoantibodies directed to nuclear autoantigens (ANA), with prevalence varying from 80 to 98% [8,9]. They represent a heterogeneous group of autoantibodies directed to various antigenic targets, and in some cases they are associated with the extent of skin involvement and with clinical outcome. The two types of ANA that are most frequently associated with SSc, namely anticentromere (ACA) and anti-topoisomerase I antibodies (ATA), are found in over 50% of SSc patients [10–13]. In addition, other autoantibodies can be detected, often producing nucleolar staining (AnoA) by indirect immunofluorescence (IIF) on HEp-2 cells. These include antibodies to fibrillarin (AFA), PM/Scl complex (anti-PM/Scl) [15], Th/To [16], human Upstream-Binding Factor (hUBF/NOR-90) [17], and to the RNA polymerase (anti-RNAP) family [18]. Other autoantibodies such as anti-U1RNP (generally associated with overlap syndrome – Sharp's syndrome), anti-Ku [19], and more recently anti-Platelet-Derived Growth Factor Receptor (PDGFR) [20,21] have been reported in SSc.

Unfortunately, the only available methods for identification of autoantibodies other than ACA and ATA are cumbersome and not suitable for routine use. For this reason, these autoantibodies are not routinely looked for in SSc patients. Recently, however, immunoassay methods for the detection of anti-RNAP III [22], anti-PM/Scl [23], and AFA [24], have been developed, which have shown that a subset of SSc patients who lack the two major SSc-related antibodies can be identified and diagnosed based on the presence of other less common antibodies. Hence, the availability of a practical and rapid assay to detect the majority of these secondary antibodies could prove very useful in diagnosing SSc patients.

The aim of this study was to evaluate the diagnostic accuracy and the clinical association of 13 different SSc-associated autoantibodies, simultaneously detected using a new multiplex line immunoblot assay (LIA).

2. Material and methods

2.1. Patients

Sera of 210 Caucasian patients with SSc (mean age 58.6 years, range 24–83; 194 females; 16 males) were consecutively collected in three different rheumatologic centers in Italy (Pordenone, Chieti, and Pescara) from 2000 to 2011. All patients fulfilled the American College of Rheumatology classification criteria for definite SSc [25] and were classified as having limited cutaneous SSc (lcSSc) or diffuse cutaneous SSc (dcSSc) according to LeRoy et al. [26]. Clinical data were collected from retrospective review of the medical records (Table 1). Esophageal involvement was assessed by double-contrast esophagography and manometry measurement. Pulmonary fibrosis was defined by radiograph or high resolution computed tomography and functional tests (forced vital capacity and diffusion lung capacity for carbon monoxide). Pulmonary arterial hypertension (PAH) was diagnosed using electrocardiography and echocardiography. The echocardiographic value of 35 mm Hg at rest was set as cut-off and patients with values >45 mm Hg were subjected to right heart catheterization. Renal function was determined by routine biological test and by echography.

Renal crisis was defined as the abrupt onset of accelerated arterial hypertension or rapidly progressive oliguric renal failure, and muscular involvement was evaluated by creatine-phosphokinase and specific electromyographic alterations.

In addition, 150 control sera from 32 patients with systemic lupus erythematosus (SLE), 25 with Sjögren's syndrome (SS), 19 with undifferentiated connective tissue disease (UCTD), 40 with rheumatoid arthritis (RA), 9 with polymyositis/dermatomyositis (PM/DM), 10 with overlap syndromes (SLE/AR; SLE/SS; AR/SS), and 15 with cryoglobulinemia were also studied. All control patients were diagnosed according to internationally recognized criteria. All sera were frozen at –80 °C until assayed. The study met and was in compliance with all ethical standards in medicine according to the Declaration of Helsinki.

2.2. Autoantibody detection

Sera were tested for ANA by IIF on HEp-2 cells (Euroimmun AG, Lübeck, Germany), and for 13 different autoantibody specificities: Ro-52, PDGFR, Ku, two sub-specificities of PM/Scl complex (PM/Scl-75 and PM/Scl-100), Th/To, NOR-90, AFA, two sub-specificities of RNAP III (RP-155 and RP-11), two sub-specificities of ACA (CENP-B and CENP-A), and ATA by using a LIA profile developed by Euroimmun. With the exception of ATA, all the autoantigens coated on the strip were recombinant proteins expressed with the baculovirus system in insect cells (CENP-A, CENP-B, PM/Scl-100, PM/Scl-5, Ku, Ro-52), in *E. coli* (RP-11, RP-155, Fibrillarin, hUBF/NOR-90, Th/To), or in mammalian cells (PDGFR). The LIA was performed following the manufacturer's instruction; blot strips were digitalized using a camera and band intensities were determined by a computer program (EUROLineScan, Euroimmun AG). Signal strengths of 6–10 units (U) were considered intermediate, and results greater than 11 U were considered positive, as recommended by the manufacturer.

Table 1
Clinical and laboratory features of SSc patients.

Clinical/laboratory features	No.	%
lcSSc	146	69.5
dcSSc	64	30.5
Gastrointestinal involvement	73	34.7
Pulmonary fibrosis	41	19.5
Pulmonary hypertension	29	13.8
Renal involvement	7	3.3
Myositis	8	3.8
Renal crisis	3	1.4
ANA positive	187	89.0
Fluoroscopic pattern:		
ACA	63	30.0
DG	41	19.5
ANoA	47	22.3
SP	25	11.9
DFS70	1	0.47
NuMA	1	0.47
SP + MND	1	0.47
ANoA + SP	8	3.8
ANoA + NuMA	1	0.47

lcSSc, limited cutaneous SSc; dcSSc, diffuse cutaneous SSc; ACA, anticentromere; DG, discrete grainy; ANoA, anti-nucleolar; SP, speckled; DFS70, dense fine speckled 70; NuMA, mitotic spindle apparatus; MND, multiple nuclear dots.

2.3. Statistical analysis

Diagnostic sensitivity and specificity, positive likelihood ratio (LR+), negative likelihood ratio (LR−) and odds ratio were calculated for each antibody assay. Chi-square test was used to determine significant differences ($p < 0.05$) between sets of categorical data. MedCalc software (Mariakerke, Belgium) was used for statistical analysis.

3. Results

One-hundred-eighty-eight (89%) of the 210 SSc patients were ANA positive and the more frequent fluorescence patterns were ACA (30%), discrete grainy (19.5%) (which is usually associated with ATA), and ANoA (22.3%). Other patterns were observed less frequently (Table 1).

Sensitivity, specificity, LR+, LR− and OR for each one of the autoantibodies detected by the multiplex LIA are shown in Table 2. In contrast to a low sensitivity of the single autoantibody, ranging from 0.48% of AFA to 30.5% of CENP-B (with the only exception of anti-Ro52; 50%), all the autoantibodies were very specific (from 93.3% of anti-PMScl-75 to 100% of anti-PDGFR, AFA and anti-RP-11). 50.5% of the SSc patients were positive for ACA or ATA, and 75.7% for at least one of the autoantibodies tested. Excluding anti-Ro52 (which is not specific for SSc) from the evaluation, the overall sensitivity decreased to 71.4%, but the specificity increased from 38.7% to 80%. The LR+, which represents the likelihood that the autoantibody positivity would be expected in a patient with SSc compared to the likelihood that the same result would be expected in a patient without SSc, was very high for ATA (30), anti-RP-11 (78.6), anti-PDGFR (14.2) and CENP-B (11.4); high for CENP-A (7.6), AFA (7.2) and anti-RP-155 (8.5); and moderate for anti-PMScl-100 (3.3). For all the other autoantibodies the LR+ was low. On the other hand, the LR− was not significant for any of the autoantibodies tested (close to 1).

Concerning the autoantibodies directed to different subunits of the same autoantigen (Fig. 1), autoantibodies to the PMScl-75 subunit

were prevalent (23/210) compared to autoantibodies directed to the PMScl-100 subunit (14/210); in 9 patients the two autoantibodies were co-present. In contrast, the prevalence of autoantibodies directed to the two different subunits of RNAP III (i.e. RP-155 and RP-11) and ACA (CENP-A and CENP-B) was very similar and the co-presence was a constant. Only one patient with SSc was positive exclusively for anti-RP-11 and anti-CENP-A, and two and four for anti-RP-155 and anti-CENP-B, respectively.

Autoantibodies present in SSc usually are mutually exclusive, as confirmed also in this study, with the important exception of anti-PMScl-75, which in seven cases was associated with ACA (Table 3).

As regards the clinical associations, ATA and anti-RP155 were significantly associated with dcSSc, whereas ACA was associated with lcSSc ($p < 0.00001$); no significant association with skin involvement has been shown for the other autoantibodies (Table 4). ATA were significantly associated with a high prevalence of pulmonary fibrosis ($p = 0.04$), whereas ACA were not significantly associated with pulmonary fibrosis ($p = 0.004$ for CENP-A and $p = 0.009$ for CENP-B) (Table 5). No association was found between any autoantibody and pulmonary hypertension, whereas a strong association was seen between anti-PMScl-100 or anti-PMScl-75 and myositis (OR 195, 95% CI: 21.03–1807.1; OR 17.1, 95% CI 3.7–77.6, respectively). Finally, no significant association was seen between any autoantibody and gastrointestinal involvement, renal involvement, or renal crisis.

4. Discussion

The significance of autoantibodies in SSc is still unclear, although there is a growing body of evidence that antibodies are not just markers of disease but they are also predictors of disease outcome and organ involvement [10,27]. However, even though multiple cohort studies have focused on the association of specific autoantibodies with clinical presentation in SSc patients, the use of different testing techniques and

Table 2

Sensitivity (SE), specificity (SP), positive likelihood ratio (LR+), negative likelihood ratio (LR−) and odds ratio (OR) of the different autoantibody specificities detected by the multiplex line immunoassay.

	SE (%) (CI 95%)	SP (%) (CI 95%)	LR+ (CI 95%)	LR− (CI 95%)	OR (CI 95%)
Ro-52	38/210 (18.1) (13.1–23.9)	75/150 (50.0) (41.7–58.2)	0.36 (0.26–0.50)	0.61 (0.51–0.55)	0.22 (0.13–0.35)
PDGFR	2/210 (0.95) (0.12–3.4)	150/150 (100) (0.96–100)	14.2 (0.02–8159)	0.99 (0.98–1.00)	1.44 (0.2–16.05)
Ku	10/210 (4.7) (2.3–8.6)	144/150 (96.0) (91.5–98.5)	1.19 (0.44–3.20)	0.99 (0.95–1.00)	1.2 (0.42–3.37)
PMScl-75	23/210 (10.9) (7.1–15.9)	140/150 (93.3) (88.1–96.7)	1.64 (0.80–3.35)	0.95 (0.89–1.00)	1.72 (0.79–3.73)
PMScl-100	14/210 (6.7) (3.6–10.9)	147/150 (98.0) (94.3–99.6)	3.33 (0.97–11.39)	0.95 (0.91–0.99)	3.5 (0.98–12.4)
Th/To	7/210 (3.3) (1.3–6.7)	148/150 (98.7) (95.2–99.9)	2.55 (0.52–11.8)	0.98 (0.95–1.00)	2.55 (0.52–12.45)
NOR-90	10/210 (4.8) (2.3–8.58)	145/150 (96.7) (92.4–98.9)	1.42 (0.49–4.09)	0.98 (0.94–1.00)	1.45 (0.48–4.33)
AFA	1/210 (0.48) (0.1–2.6)	150/50 (100) (96.3–100)	7.2 (0.01–4737)	0.99 (0.98–1.00)	0.72 (0.04–6.97)
RP-155	12/210 (5.7) (2.9–9.8)	149/150 (99.3) (96.3–99.9)	8.57 (1.12–65.2)	0.95 (0.91–0.98)	9.03 (1.16–70.27)
RP-11	11/210 (5.2) (2.6–9.2)	150/150 (100) (96.3–100)	78.5 (0.16–39602)	0.95 (0.92–0.98)	5.78 (1.14–32.12)
CENP-B	64/210 (30.5) (24.3–37.2)	146/150 (97.3) (93.3–99.3)	11.4 (4.25–30.7)	0.71 (0.65–0.78)	16.0 (5.67–45.0)
CENP-A	61/210 (29.5) (23.0–35.7)	144/150 (96.0) (91.5–98.5)	7.26 (3.22–16.36)	0.74 (0.67–0.81)	9.14 (3.95–21.18)
ATA	42/210 (20.0) (14.8–26.1)	149/150 (99.3) (96.3–99.9)	30.0 (4.17–215.6)	0.80 (0.75–0.86)	25.14 (4.86–129.9)
Overall	159/210 (75.7) (69.3–81.3)	58/150 (38.7) (30.8–46.9)	1.23 (1.06–1.43)	0.63 (0.46–0.86)	1.95 (1.24–3.08)
Overall (without Ro-52)	150/210 (71.4) (64.8–77.1)	120/150 (80.0) (72.7–86.1)	3.57 (2.56–4.97)	0.36 (0.28–0.45)	10.0 (6.06–16.48)

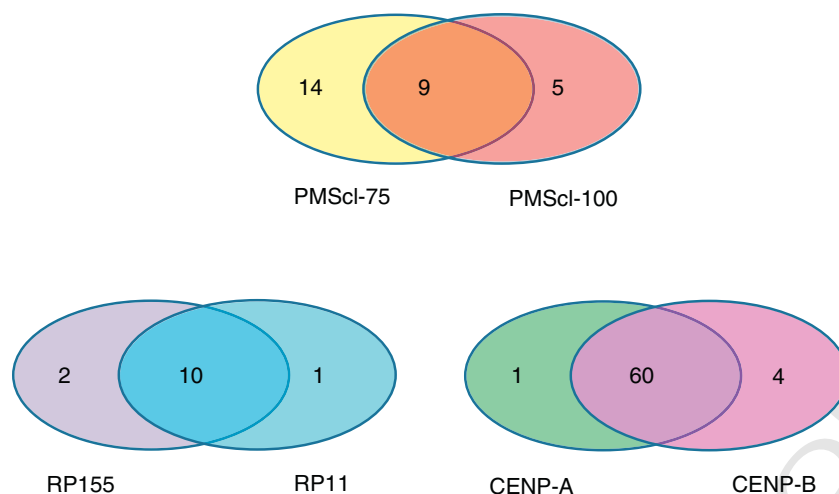


Fig. 1. Venn diagrams showing the co-presence of autoantibodies binding different antigenic specificities of the PM-Scl complex, RNAP-III complex and anti-centromere complex.

their lack of standardization as well as the variable definition of organ involvement make the interpretation difficult [28,29].

In a systematic review of the literature using standards for studies of diagnostic tests [30,31], the American College of Rheumatology (ACR) ad hoc Committee on Immunologic testing guidelines [27] reported that the overall diagnostic sensitivity of ACA (detected by IIF) comparing SSc patients with those with other connective tissue diseases (CTD) was 31% and the specificity was 97.4% (LR + 12.5 and LR − 0.7). These results, which are very similar to those obtained in our study, show that this autoantibody is very useful for the diagnosis of SSc. Besides, our study confirms that the performance of recently developed immunoassays using a CENP-B recombinant protein is very similar to those of IIF on HEp-2 cells. In a previous study [32] it was reported that anti-CENP-A represents a sensitive, specific and independent marker for the detection of ACA and that when both anti-CENP-A and anti-CENP-B are positive, the sensitivity for ACA decreases, but the specificity increases significantly (99.3% vs 94.8% of anti-CENP-B alone). Our study only partially confirms this data, since a positive association of anti-CENP-A and anti-CENP-B was present only in 28.5% of SSc patients (vs 30.5% of anti-CENP-B alone), but the specificity showed a very small increase (98% vs 97.3% of anti-CENP-B alone). Therefore, we believe that further studies are needed to verify the usefulness of CENP-A detection.

In the ACR systematic review, ATA had an overall sensitivity of 26%, a specificity of 99.5%, and a LR + of 52 in comparison with 20%, 99.3%, and 30, respectively, of our study, confirming the high predictive value of this autoantibody for the diagnosis of SSc [33].

Concerning the clinical correlation, our data substantially confirmed those of ACR, expressed as sensitivity (44%), specificity (93%), and LR + (6.1) of ACA in predicting the presence of lcSSc, whereas

more robust resulted in our study than in the ACR study of the correlation of ATA with dcSSc (sensitivity 50% vs 37%; specificity 93% vs 82%; LR + 7.30 vs 2.0). However, it is important to remark that in the ACR systematic revision the majority of the studies included used the immunodiffusion method for ATA detection, a method that is no longer used in clinical laboratories. Finally, using the LIA we confirmed that ACA was associated with a significantly lower likelihood of radiographic pulmonary fibrosis (LR + 0.4; $p = 0.009$) and that ATA was positively associated with radiographic pulmonary fibrosis, even if the association is not strong (LR + 1.9; $p = 0.048$). Therefore, our results support the conclusions of the ACR study that ACA are very useful in predicting a limited skin involvement and a lesser likelihood for the development of pulmonary fibrosis; while ATA are useful in predicting a greater likelihood of diffuse skin involvement and pulmonary fibrosis.

It is clear, as we have mentioned, that ACA and ATA are important for the diagnosis and prognosis of SSc, however they are positive in only about 50–55% of patients. In ACA- and ATA-negative SSc patients, other autoantibodies may be found. In 15–40% of patients ANoA have been reported [34,35] and the singular autoantibody specificities accountable for ANoA pattern in IIF seem to play a role in disease classification and in prediction of organ involvement [36,37]. Unfortunately, up to now research of these antibodies has been confined to a small number of research laboratories, since the techniques used for their detection (i.e. immunoprecipitation) are laborious and time-consuming. For this reason, given the low prevalence of the ANoA specificities in SSc, and the variations between different ethnic groups of the frequency of these autoantibodies, no conclusive data on their diagnostic and prognostic value are available.

Table 3
Autoantibody co-presence in patients with SSc.

	PDGFR	Ku	PMScl75	PMScl100	Th/To	NOR90	AFA	RP155	RP11	CENP-B	CENP-A	ATA
PDGFR	2											
Ku	–	10										
PMScl75	–	–	23									
PMScl100	–	–	9	14								
Th/To	–	–	3	–	7							
NOR90	–	1	1	1	–	10						
AFA	–	–	–	–	–	1	1					
RP155	–	1	1	–	–	–	–	12				
RP11	–	1	1	–	–	–	–	10	11			
CENP-B	–	1	7	1	1	–	–	–	–	64		
CENP-A	–	1	7	1	–	–	–	–	–	60	61	
ATA	–	2	1	–	1	–	–	1	1	–	1	42

Table 4

Sensitivity (SE), specificity (SP), odds ratio (OR), positive likelihood ratio (LR+), and likelihood ratio chi square (χ^2) of the tested autoantibodies in predicting cutaneous involvement in SSC.

		lcSSc (146)	dcSSc (64)	SE% ^a	SP% ^a	OR ^a	LR+ ^a	χ^2	p
	Ro-52	28	10	19.1	77.2	0.80	0.84	0.27	0.60
	PDGFR	0	2	3.1	100	4.7	4.5	1.7	0.10
	Ku	6	4	4.1	93.7	0.64	0.65	0.43	0.51
	PMScl-75	16	7	10.0	89.0	1.0	1.0	0.00	0.99
	PMScl-100	10	4	6.8	93.7	1.1	1.09	0.03	0.87
	Th/To	6	1	4.1	98.4	2.7	2.63	1.03	0.31
	NOR-90	9	1	6.2	98.4	4.13	3.94	2.52	0.11
	AFA	1	0	0.72	98.4	0.45	0.46	0.29	0.59
	RP-155	5	7	10.9	96.6	3.46	3.19	4.66	0.03*
	RP-11	5	6	9.4	96.6	2.91	2.73	2.90	0.08
	CENP-B	61	3	41.8	95.3	14.6	8.91	35.6	0.0000*
	CENP-A	58	3	39.7	95.3	13.4	8.47	26.5	0.0000*
	ATA	10	32	50	93.1	13.6	7.30	48.5	0.0000*

^a Data are relative to the capability of Ro-52, Ku, PM-Scl 75, PM-Scl 100, Th/To, NOR 90, AFA, CENP-B and CENP-A in predicting limited cutaneous involvement (lcSSc), and of PDGFR, RP 155, RP11 and ATA in predicting diffuse cutaneous involvement.

* p<0.05.

In recent years, many advances have been done on the knowledge of the structure of autoantigenic molecules and of the immunodominant epitopes targeted by this autoantibody. As a consequence, recombinant proteins of the main autoantigenic subunits of RNP, PMScl, AFA, hUBF/NOR-90, Th/To are now available, and commercial assays have been developed using these proteins as substrate [22–24,38,39]. The multiplex LIA used in our study allowed the simultaneous detection of several antibodies, including ANoA specificities. The parallel detection of all the autoantibodies brought the diagnostic sensitivity to 71.4% (excluding from the calculation anti-Ro-52 which is not a specific marker of SSC). It should be remarked that if multiple tests are carried out, a sizeable number of false positive results are likely, purely by chance [40]. Therefore, in our multiplex assay, even though the specificity of each individual autoantibody tested was very high, the overall specificity decreases to 80%.

4.1. Anti-RNAP III antibodies

Concerning the single autoantibodies, the reactivity toward RNAP III, and specifically against the immunodominant epitope present in the largest subunit of RNAP (RP-155), was of 5.7%, similar to that reported in other studies on French [41] or Italian [42,43] patients, but less prevalent than that reported in American or Japanese populations [22,41]. In 3/12 cases anti-RNAP III (RP-155) was associated with other autoantibodies: 1 with ATA, 1 with Ku, and 1 with PMScl-75, showing that this autoantibody is not fully mutually

Table 5

Use of autoantibodies in predicting pulmonary fibrosis in SSC.

		Sensitivity (%)	Specificity (%)	OR	LR+	χ^2	p
	Ro-52	13/41 (31.7)	131/169 (77.5)	1.60	1.41	1.46	0.22
	PDGFR	2/41 (4.9)	168/169 (99.4)	8.61	8.24	3.21	0.07
	Ku	1/41 (2.4)	160/169 (94.7)	0.44	0.46	0.70	0.40
	PMScl-75	5/41 (12.2)	151/169 (89.3)	1.16	1.14	0.08	0.77
	PMScl-100	2/41 (4.9)	157/169 (92.9)	0.67	0.68	0.28	0.59
	Th/To	3/41 (7.3)	165/169 (97.6)	3.25	3.09	2.06	0.15
	NOR-90	1/41 (2.4)	160/169 (94.7)	0.44	0.46	0.70	0.40
	AFA	1/41 (2.4)	169/169 (100)	2.03	2.01	0.30	0.58
	RP-155	2/41 (4.9)	159/169 (94.0)	0.81	0.82	0.07	0.79
	RP-11	3/41 (7.3)	161/169 (95.3)	1.59	1.55	0.41	0.52
	CENP-B	6/41 (14.6)	111/169 (65.7)	0.32	0.42	6.72	0.009 ^a
	CENP-A	5/41 (12.2)	113/169 (66.8)	0.28	0.37	8.0	0.004 ^a
	ATA	12/41 (29.3)	143/169 (84.6)	2.27	1.90	3.91	0.048 ^b

^a Negative significant association.

^b Positive significant association.

exclusive with other autoantibodies. Apparently, this finding may be in contrast with previous studies [22,44] which considered only ACA and ATA. Autoantibodies against the RP-11 subunit of RNAP III, despite having a specificity of 100%, were less sensitive than anti-RP-155 and therefore they do not seem to add diagnostic value to anti-RP-155. We confirmed that anti-RP-155 positivity predicts dcSSc (LR+ 3.19), but we did not find any association with clinical parameters, and in particular with renal crisis. This finding, however, may depend on the low number of SSC patients with renal crisis in our series.

4.2. Anti-PMScl antibodies

Anti-PMScl autoantibodies are a heterogeneous group of autoantibodies directed to several proteins of the nucleolar PMScl macromolecular complex, and the two main autoantigenic subunits have been identified and termed PMScl-100 and PMScl-75 [45,46]. According to previous studies indicating PMScl-100 as the main target of anti-PMScl reactivity, the majority of commercially available assays use recombinant PMScl-100 protein as autoantigen. In contrast, other researchers reported that the percentage of patients presenting anti-PMScl-75 antibodies exceeds that of anti-PMScl-100 antibodies [47]. This was confirmed in a recent study on a large cohort of German patients with SSC [39], where the diagnostic sensitivity of PMScl was of 12.5%, and among anti-PMScl positive 10.4% and 7.1% were positive for anti-PMScl-75 and anti-PMScl-100, respectively. These results are very similar to those obtained in our study and demonstrate that when assays based only on PMScl-100 are used, many cases of reactivity against the PMScl complex can be missed. Both the autoantibody specificities were strongly associated with the presence of myositis (polymyositis/scleroderma overlap), but unlike Hanke and coworkers, we did not find any significant association between anti-PMScl-75 and dcSSc. On the contrary, 7/23 PMScl-75 positive patients were also ACA-positive, a marker strongly associated with lcSSc.

4.3. Anti-fibrillarin antibodies

Even if the prevalence of AFA has been shown to be higher in Afro-Caribbeans than in other ethnic groups [14,48,49], only one SSC patient (0.48%) was positive in our study, a lower percentage than expected on the basis both of previous studies on European population [41,43] and of a previous study by our group, where the prevalence of AFA in ACA- and ATA-negative SSC patients was 10% [24]. Even if we cannot exclude that this difference could be related to the cohort of patients evaluated, a possible explanation may reside on the analytical method used. In fact, previous studies used immunoprecipitation or immunoenzymatic assays with full-length recombinant proteins expressed in baculovirus, whereas in the LIA assay a recombinant protein expressed on *E. coli* is used. Other studies comparing the results of different methods in the same population are needed to explain this difference. Because of the low number of positive patients, we did not look for clinical correlation with AFA.

4.4. Anti-Th/To antibodies

Anti-Th/To antibodies recognize a complex of RNA-associated endonucleases, which have been identified as the human MRP RNase and the P ribonuclein RNase complexes [50], which are considered to be fairly specific for SSC. In our cohort the prevalence of anti-Th/To was of 3.3%, consistent with the prevalence found in other studies among Caucasians [41,51], and in particular in Italian patients, as recently shown by Ceribelli et al. [52] using the immunoprecipitation method. In the latter study, anti-Th/To positive patients had mild cutaneous disease, mild pulmonary fibrosis and more frequently developed pericarditis. The prognosis, however, appeared excellent. In our study, all but one showed lcSSc. The patient with dcSSc was also ATA positive. No significant correlation was found with pulmonary fibrosis or pulmonary hypertension, confirming the results of Gliddon et al. [53]. We confirmed

the high specificity for SSc of anti-Th/To antibodies (98.7%), since only one patient with PM/DM and one with MCTD were Th/To positive.

4.5. Anti-Ku antibodies

Although the anti-Ku prevalence is rather low in SSc patients, it is not homogeneous, depending on several features such as disease type, genetic and geographical clustering, and also method of detection, and it may increase to 55% in polymyositis/SSc overlap [54]. In our cohort, the prevalence of anti-Ku (4.7%) was higher than in other studies on European population [41,55], and in 4 of 10 positive patients this autoantibody has been found associated with another autoantibody (anti-hUBF/NOR-90, anti-RNAP III, ACA, ATA, respectively). As largely expected, anti-Ku was less specific for SSc than the majority of autoantibodies tested, because it was positive in 12.4% of SLE, 5.2% of UCTD, and 2.5% of RA. Differently from other studies, we did not show any correlation with musculoskeletal manifestations, but again it may depend on the population evaluated.

4.6. Anti-hUBF/NOR-90 antibodies

Despite hUBF/NOR-90 is not considered highly specific for SSc, nine patients in our series were positive only for this autoantibody. Thus, based on our findings, this antibody may prove to be a useful serological marker in patients who test negative for ATA, ACA and anti-RNAP III. hUBF/NOR-90 was prevalently associated with lcSSc (9 of 10 patients), confirming the results of a recent review [56]. However, this point remains uncertain as no large cohort studies on the clinical association of this autoantibody are available.

4.7. Anti-PDGF antibodies

PDGFs are potent stimulants of cell proliferation and migration, and PDGF signaling plays an important role in wound healing, angiogenesis, inflammation, vascular remodeling, and fibrosis [57]. Recently, Baroni et al. [12] have reported the existence of stimulatory anti-PDGF antibodies in sera of patients with SSc, suggesting that these autoantibodies are involved in the pathogenesis of scleroderma. They have been able to find these autoantibodies in all the patients with SSc, but not in the controls. Conversely, other workers [58,59] could not demonstrate the presence of stimulatory anti-PDGF autoantibodies in patients with SSc. Using an immunoenzymatic (ELISA) method, Balada et al. [60] reported the presence of these autoantibodies in 32.7% of patients with SSc and in 34.3% of healthy controls, and Kurasawa et al. [61] in 21% of patients with SSc and in 29% of patients with SLE. In our study, in spite of the absolute specificity, only two SSc patients (0.95%) were anti-PDGF positive. The discrepancy reported in the various studies is probably due to method-dependent variability, and in particular to the results obtained with very sensitive ELISA methods that may detect low-avidity naturally occurring autoantibodies. More studies are needed to verify this hypothesis, and a consensus on the best assay to detect anti-PDGF autoantibodies has to be obtained before proposing this antibody as a diagnostic marker of SSc.

In conclusion, the possibility of detecting simultaneously a broad spectrum of SSc-associated autoantibodies by a multiplex assay increases diagnostic sensitivity, allowing for finding a positive serological marker in about 50% of ACA- and ATA-negative SSc patients. We confirm the good diagnostic performance of anti-RNAPIII and anti-PMScl antibodies, and the high specificity of AFA and anti-Th/To antibodies. The multiplex LIA assay can be a valid substitute to immunoprecipitation tests in routine practice and, due to the wide range of antibodies detectable in a single step, may represent a more efficient laboratory approach to SSc diagnosis. However, with the increasing number of diagnostic assays for testing SSc-related autoantibodies, more diligent attention needs to be given to the standardization of the assays and to the analytical platforms in which they are employed. To this end, the

availability of international reference sera for all these antibodies could be an important step toward standardization.

Take-home messages

- There is a growing body of evidence that antibodies in SSc are not just markers of disease but they are also predictors of disease outcome and organ involvement.
- New serological assays for detection of autoantibodies different from ACA and ATA in SSc are now available.
- LIA assay could be a more practical and efficient method than traditional immunoprecipitation to detect antinuclear autoantibodies in routine practice, greatly improving sensitivity in SSc diagnosis.
- With the increasing number of diagnostic assays to test SSc-related autoantibodies, more diligent attention needs to be given to the standardization of the assays.

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