Anti-Th/To Are Common Antinucleolar Autoantibodies in Italian Patients with Scleroderma

ANGELA CERIBELLI, ILARIA CAVAZZANA, FRANCO FRANCESCHINI, PAOLO AIRÒ, ANGELA TINCANI, ROBERTO CATTANEO, BRAD A. PAULEY, EDWARD K.L. CHAN, and MINORU SATOH

ABSTRACT. ***Objective.*** Patients with scleroderma (systemic sclerosis; SSc) can be classified into subsets based on autoantibody profile and clinical features. Specificities such as anti-Th/To and anti-fibrillarin (U3RNP) are detectable mainly by immunoprecipitation (IP), which is not widely used in clinical laboratories. We examined the autoantibody profiles and clinical manifestations in a cohort of Italian patients with SSc, focusing on anti-Th/To and anticentromere (ACA) antibodies, associated with limited cutaneous SSc (lcSSc).

Methods. Sera from 216 consecutive patients with SSc were tested for ACA (by indirect immunofluorescence), antitopoisomerase I (topo I; by counterimmunoelectrophoresis), and anti-RNA polymerase III (RNAPIII; by ELISA). Forty-one sera negative for these specificities were tested by IP analysis of proteins (^{35}S-methionine labeled K562 cell extract) and RNA (silver staining).

Results. Among 216 SSc patients analyzed, anti-topo I, ACA, and anti-RNAPIII were detected in 38% (81/216), 31% (67/216) and 7% (15/216), respectively. Among 41 sera negative for ACA, anti-topo I, and anti-RNAPIII and which were tested by IP, 14 were nucleolar stain-positive. Eight out of 14 (57%) showed anti-Th/To reactivity, but no anti-U3RNP was found. In comparison with ACA-positive patients, anti-Th/To-positive patients were younger ($p = 0.0046$) and more commonly were male ($p = 0.0006$). All 8 anti-Th/To-positive and all but one ACA-positive patients had lcSSc. Interstitial lung disease (ILD) and pericarditis were more frequent in anti-Th/To-positive patients.

Conclusion. Anti-Th/To are common in antinucleolar antibody-positive Italian patients with SSc. Anti-Th/To and ACA patients had lcSSc, with excellent prognosis. The anti-Th/To group had frequent pericarditis and ILD, although impairment of pulmonary function appeared mild. (First Release August 1 2010; J Rheumatol 2010;37:2071–5; doi:10.3899/jrheum.100316)

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Scleroderma (systemic sclerosis, SSc) is a chronic autoimmune connective tissue disease characterized by vascular changes, fibrosis, and presence of autoantibodies, such as anticentromere (ACA), antitopoisomerase I (topo I), and anti-RNA polymerase III (RNAPIII) antibodies[1]. Patients with scleroderma can be classified into subsets associated with unique clinical features (cutaneous and visceral involvement, rapidity of disease progression, and prognosis) and specific autoantibodies. In particular, 2 major subsets, the limited (lcSSc) and the diffuse (dcSSc) cutaneous variants, are clinically recognized: dcSSc is frequently associated with anti-topo I or anti-RNAPIII antibodies, while lcSSc is associated with ACA or anti-Th/To antibodies[1,2].

In immunofluorescence antinuclear antibody (ANA) screening, anti-topo I and anti-RNAPIII show nuclear staining in fine speckled-homogeneous (topo I) or coarse speckled (RNAPIII) patterns. ELISA and other methods are widely used in clinical practice to confirm these specificities[3]. ACA that appear as a discrete speckled nuclear staining pattern can be assessed by a screening ANA test alone in most cases. In contrast, some SSc patients' sera show a pure or dominant nucleolar staining pattern. Anti-Th/To, fibrillarin (U3RNP), and PM-Scl are the common specificities of nucleolar stain-positive SSc sera. However, these autoantibodies can be identified mainly by immunoprecipitation (IP), which is not widely used in clinical laboratories. Thus, clinical information available on these antibodies is based on studies from a few institutions, despite data suggesting a significant effect of ethnicity on autoantibody profile and clinical features[1].

We examined the clinical and immunological features

From the Rheumatology Unit, A.O. Spedali Civili, Università degli Studi, Brescia, Italy; and Department of Oral Biology and Department of Medicine, University of Florida, Gainesville, Florida, USA.

A. Ceribelli, MD; I. Cavazzana, MD; F. Franceschini, MD; P. Airò, MD; A. Tincani, MD; R. Cattaneo, MD, Rheumatology Unit, A.O. Spedali Civili di Brescia, Università degli Studi di Brescia; B.A. Pauley, BSc; E.K.L. Chan, PhD, Department of Oral Biology, University of Florida; M. Satoh, MD, PhD, Department of Medicine, University of Florida.

Address correspondence to Dr. A. Ceribelli, Department of Oral Biology, College of Dentistry, University of Florida, PO Box 100424, Gainesville, FL 32610-0424. E-mail: dott.ceribelli@libero.it

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associated with specific SSc-related autoantibodies in an Italian cohort of patients with SSc, focusing on analysis of sera that were nucleolar stain-positive by IP. Moreover, since anti-Th/To was the main antinucleolar specificity in our SSc sera cohort, and anti-Th/To and ACA are commonly associated with the limited variant of SSc, their clinical manifestations were compared.

MATERIALS AND METHODS

Patients. Two hundred sixteen consecutive patients followed at our institution (Rheumatology Unit, Spedali Civili, Brescia, Italy) during the period 2000 to 2008, who had SSc diagnosed and classified into clinical subsets according to the LeRoy criteria[4], were studied[5]. They were all Caucasians of Italian ancestry. Clinical and laboratory data were collected from medical records. Skin thickness was evaluated with the modified Rodnan Skin Score (RSS) at the initial visit, during followup, and at the last visit. Puffy hands were defined as an increase in soft tissue mass due to dermal imbibition, particularly at the fingers, with abolishment of skin contours and skin folds. Pulmonary function tests (PFT) with evaluation of forced vital capacity (FVC) and diffusion lung capacity for carbon monoxide (DLCO) were performed at the time of diagnosis, and at least yearly during the followup. Interstitial lung disease (ILD) was defined by chest radiograph or high resolution computed tomography. The latter was performed in patients with unexplained dyspnea or abnormal PFT. Pericarditis was diagnosed by clinical examination and echocardiography. Pulmonary arterial hypertension (PAH) was diagnosed by right-heart catheterization, for values > 25 mm Hg of mean pulmonary arterial pressure at rest[6]. Right-heart catheterization was performed in patients who had high systolic pressure (> 45 mm Hg) on Doppler echocardiography screening test, or who had unexplained dyspnea. No other potential cause for pulmonary hypertension (such as left-heart disease, pulmonary disease, chronic thromboembolism) was detected. One serum sample from each patient was tested for autoantibodies. The study protocol was approved by the Institutional Review Board. The study met and was in compliance with all ethical standards in medicine and informed consent was obtained from all patients according to the Declaration of Helsinki.

Indirect immunofluorescence (IIF). Antinuclear/cytoplasmic autoantibodies in sera were tested using commercial HEp-2 slides (BioRad, Hercules, CA, USA; Inova Diagnostics, San Diego, CA, USA) and they were considered positive at titer ≥ 1:160.

Counterimmunoelectrophoresis (CIE). CIE was performed using rabbit thymus and human or porcine spleen extracts as substrates[7]. Specificities including antibodies to topo I, PM-Scl, Ku, U1RNP, Ro/SSA, La/SSB, Jo-1, Sm, PCNA, ribosomal P, Ki/SL, and Mi-2 were determined using reference sera.

Anti-RNAPIII ELISA. Anti-RNAPIII antibodies were tested using a commercial ELISA kit (Inova Diagnostics).

Immunoprecipitation (IP). Forty-one sera that were negative for anti-topo I (CIE), RNAPIII (ELISA), and ACA (IIF) were tested by IP. Protein analysis was performed by IP of ^{35}S-methionine radiolabeled K562 cell extract, SDS-polyacrylamide gel electrophoresis (PAGE), and autoradiography. RNA analysis was done with urea-PAGE and silver staining (Silver stain plus; Bio-Rad)[8]. Specificities were verified using reference sera.

Statistical analyses. All variables were analyzed by Fisher exact test or chi-square test for prevalence. Student's t test, Mann-Whitney, or Wilcoxon test was used for analyses of levels/values between groups, using Prism 5.0 for Windows (GraphPad Software, San Diego, CA, USA). Statistical significance was accepted at $p < 0.05$.

RESULTS

Laboratory data. Among 216 SSc patients analyzed, anti-topo I, ACA, and anti-RNAPIII were detected in 38% (81/216), 31% (67/216), and 7% (15/216), respectively. Anti-RNAPIII by ELISA was also positive in 1 of 67 ACA-positive and 3 of 81 anti-topo I-positive sera; however, with a significantly lower level in the double-positive samples[5]. Fifty-three SSc patients were negative for these, and 41 available sera were tested further by IP. Among these, 14 (34%) had a nucleolar pattern, 22 (54%) had speckled and/or homogeneous nuclear pattern, and 5 (12%) sera were negative for ANA by IIF. RNA components immunoprecipitated by these 41 sera were also analyzed. Among the 14 antinucleolar antibody-positive sera, 8 immunoprecipitated 7-2 and 8-2 RNA (Figure 1A) and were identified as anti-Th/To (8/14; 57%). The nucleolar patterns by anti-Th/To-positive sera were either isolated or associated with a nuclear speckled pattern (Figure 1B). One anti-NOR90 and one anti-topo I (previously negative by CIE) were also found in this group. Considering sera with speckled and/or homogeneous pattern, 2 positive for anti-RNAPII (without anti-RNAPI/III), one for anti-PL7, and one for anti-Ro/SSA (which was negative by CIE) were detected. Apart from the double positivity in the 4 anti-RNAPIII samples, no patient had more than one SSc-specific autoantibody, and we did not detect any anti-fibrillarin (U3RNP) in the 41 sera tested by IP, including 14 antinucleolar antibody-positive sera.

Clinical data. Since anti-Th/To and ACA are the specificities associated with lcSSc, clinical features of 8 anti-Th/To-positive patients were compared with those of 67 ACA-positive patients (Table 1). In the anti-Th/To-positive group, patients were more frequently males (female:male 5:3 vs 66:1 for ACA-positive; $p = 0.0006$ by Fisher exact test) and they were younger ($p = 0.0046$ by t test) than ACA-positive patients. All patients in both groups had lcSSc, except for one case of dcSSc in the ACA group. Skin thickening during followup did not change significantly as evaluated by the RSS in these 2 groups. As shown in Table 1, ACA patients more often had telangiectasia ($p = 0.048$), while no difference was detected for puffy hands, calcinosis, and digital ulcers. Pericarditis ($p = 0.028$) and ILD ($p = 0.044$) were more common in anti-Th/To-positive than in ACA-positive patients. Accordingly, FVC at the time of the first evaluation as well as at the last visit was lower in anti-Th/To-positive patients than in ACA-positive patients ($p = 0.0183$ and $p = 0.0298$, respectively, Mann-Whitney test). DLCO diminished significantly over time in the ACA group, while in the anti-Th/To group there was a trend to reduction of DLCO that was not statistically significant ($p = 0.0925$; Table 1), likely due to the small number of patients in this group. However, lung involvement in anti-Th/To-positive patients was mild, as only one anti-Th/To-positive patient received immunosuppression with intravenous cyclophosphamide for pulmonary indications. The prevalence of PAH confirmed by right-heart catheterization was not statistically different between the 2

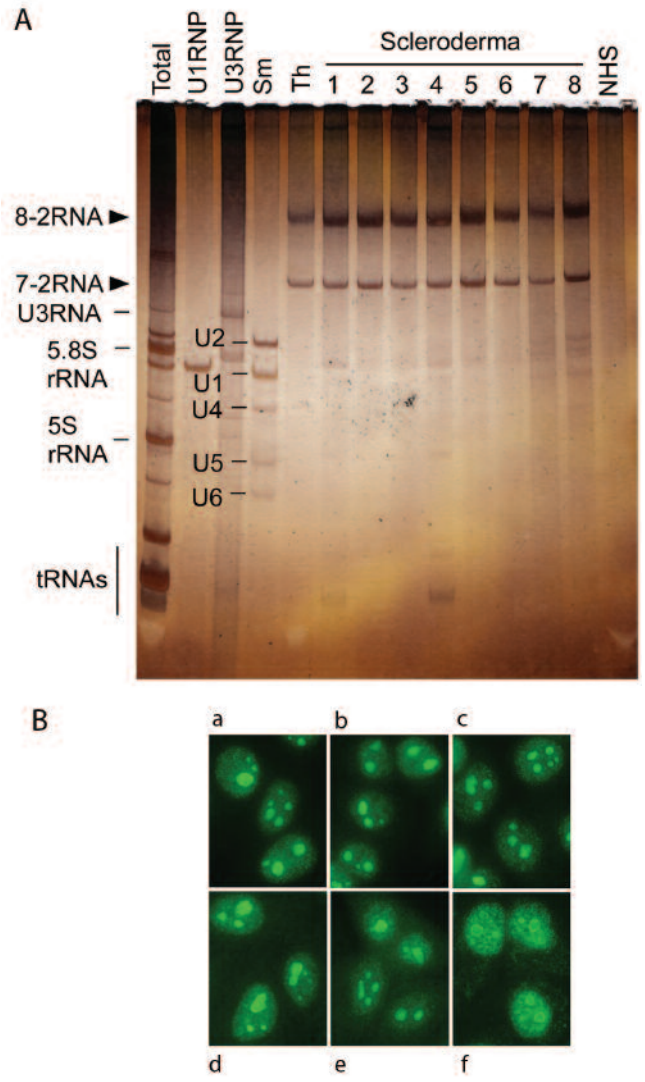

Figure 1. Immunoprecipitation and immunofluorescence in anti-Th/To positive sera. A. Immunoprecipitation analysis of RNA components. K562 cell extract was immunoprecipitated by human sera and RNA were extracted and analyzed by urea-PAGE and silver staining. Anti-Th/To antibodies were confirmed by immunoprecipitation of 7-2 and 8-2 RNA (lanes 1–8). Patterns of total RNA in cell extract (lane total), reference sera (U1RNP, U3RNP, Sm, and Th) and normal human serum (NHS) are also shown. B. Indirect immunofluorescence (IIF). The nucleolar IIF pattern of 6 of the 8 positive anti-Th/To samples is shown.

groups (Table 1): 7 cases in the ACA-positive group had PAH (7/67, 10%), and none in the anti-Th/To-positive group. Survival was excellent in both groups: only 2 patients in the ACA group died during followup, and 2 in the same group were lost to followup. The average followup was 14 years (SD ± 8) in the ACA group and 9 years (SD ± 6) in the anti-Th/To group.

DISCUSSION

In our study, anti-Th/To antibodies were detected in 8 SSc patients through the use of RNA-IP. Despite the low number of cases, their main features have been compared with those of 67 SSc patients with ACA.

Anti-Th and To antibodies were reported in 1982 and in 1983 independently, but their identity was confirmed later[9,10,11,12]. They recognize a complex of 6 proteins associated with 7-2 and 8-2 RNA, which have been identified as 2 endonucleases, the human RNase MRP and RNase P ribonucleoprotein complexes[13,14]. Since the target proteins are not readily observed in protein IP using standard ^{35}S-methionine-labeled cell extract, the specificity is usually defined based on the detection of 7-2 and 8-2 RNA components in IP[2,3].

Anti-Th/To antibodies usually show the nucleolar staining pattern in an IIF ANA test[1,2,3]. It is one of the common specificities associated with nucleolar pattern in patients with SSc, along with anti-fibrillarin (U3RNP) and PM-Scl. In our cohort of Italian patients with SSc, anti-Th/To was found in 3.7% of patients and it was very common among antinucleolar antibody-positive SSc (8/14; 57%), whereas none had anti-fibrillarin (U3RNP). This is consistent with the high prevalence of anti-Th/To in antinucleolar antibody-positive Caucasian North American SSc patients[1]. Anti-Th/To antibodies are considered to be fairly specific for SSc, because they are present in patients with SSc and primary Raynaud's phenomenon, but not in those with systemic lupus erythematosus, polymyositis/dermatomyositis, or undifferentiated connective tissue disease[10]. However, the disease specificity has been challenged by some investigators. Yamane, *et al* detected anti-Th/To antibodies in 3 of 70 patients with localized scleroderma[15]. Although one study reported that 5% (13/285) of patients classified as having idiopathic pulmonary fibrosis had anti-Th/To, 4 of them had skin changes consistent with lcSSc and 9 met the proposed criteria of the variant called "SSc sine scleroderma"[16]. In another study, 7 of 21 anti-Th/To-positive sera were from patients without SSc[17].

The prevalences of SSc-related autoantibodies in the studies that include anti-Th/To antibodies are summarized in Table 2. In all the studies, anti-Th/To antibodies are detected by RNA-IP. In studies on SSc autoantibodies, ethnicity is usually classified into relatively large categories such as Caucasian, African American, Latin, and Asian. However, "Caucasian" may be quite heterogeneous, or environmental and other factors may have significant effects, as indicated by the low prevalence of anti-RNAPIII in France (4%)[18] and in the current study in Italy (7%) compared to the high prevalence (~25%) of this specificity in Caucasians in the United States[18], Canada[19], the UK[20], and Denmark[21]. In contrast, the prevalence of anti-Th/To among Caucasians seems consistent, i.e., 2% to 5% in most studies[10,18,21], including the present study (Table 2).

The clinical significance of anti-Th/To in SSc is based on studies from only a few institutions due to the limited availability of anti-Th/To testing[3]. Our study is the first descrip-

Table 1. Demographic and clinical features in patients with SSc with anti-Th/To and anticentromere antibodies (ACA).

Characteristic	Anti-Th/To, n = 8	ACA, n = 67	p
Demographic			
Female:male	5:3	66:1	0.0006
Mean age, yrs (± SD)	54.5 (± 17.9)	66.6 (± 10.1)	0.0046
Mean disease duration, yrs (± SD)	8.5 (± 6.5)	8.7 (± 5.9)	NS
Limited SSc (lcSSc), %	100	98.5	NS
Mean time from Raynaud's phenomenon to SSc onset*, yrs (range)	6 (1–24)	9 (1–47)	NS
Skin involvement, %			
Telangiectasia	12.5	49	0.048
Puffy hands	62.5	28	NS
Digital ulcers	50	55	NS
Capillaroscopic changes	100	79*	NS
Calcinosis	12.5	28	NS
Cardiopulmonary involvement, %			
Pericarditis	25	4.5	0.028
Interstitial lung disease (by chest radiograph)	38	4.5	0.044
Pulmonary arterial hypertension	0	10	NS
Pulmonary function tests			
FVC onset, mean % ± SD	95.3 ± 18.8	114.1 ± 18.6	0.0183
Last FVC, mean % ± SD	97.2 ± 20.5	114.7 ± 20.2	0.0298
DLCO onset, mean % ± SD	81.1 ± 17.2	74.4 ± 18.9	NS
Last DLCO, mean % ± SD	68.7 ± 14.5	64.6 ± 16.3	NS
% of DLCO at onset vs at the last evaluation	p = 0.0925	p < 0.0001	

* Defined by onset of first non-Raynaud feature of SSc disease. NS: nonsignificant. FVC: forced vital capacity; DLCO: diffusion capacity for carbon monoxide.

Table 2. Prevalence of anti-Th/To and other SSc-related autoantibodies in different ethnic groups. Anti-Th/To antibodies were detected by RNA-immunoprecipitation in all the studies shown in the table.

	Kipnis 1990[24]	Okano 1990[10]	Jacobsen 2001[21]	Kuwana 2002[17]	Meyer 2007[18]	Meyer 2007[18]	Hamaguchi 2008[25]	Ceribelli 2010
No.	84	371	174	303	127	247	203	216*
Race	NA	NA	Caucasian	Japanese	Caucasian 91%	Caucasian 92%	Japanese	Caucasian
Country	USA (New Haven)	USA (Pittsburgh 1984–8)	Denmark	Japan	France	USA (Pittsburgh 1986–8)	Japan	Italy
Autoantibody, %								
Centromere	21	—	37	17	18	21	38	31
Topo I	18	—	13	30	35	22	33	38
RNAPIII	7	—	22	—	4	25	6	7
Th/To	13	4	2.3	4.8	1	4	3	3.7*
U3RNP (fibrillarin)	7	—	4.6	—	2	2	2	0*
PM-Scl	5	—	—	—	6	3	—	—
U1RNP	10	—	4	—	9	7	5	—
Ku	2	—	—	—	2	1	—	—

* Only sera without ACA, topoisomerase I, or RNAPIII were tested for anti-Th/To and U3RNP. NA: not available.

tion of anti-Th/To antibodies in a cohort of Italian SSc patients, and their clinical features were compared with patients with ACA. However, the findings must be considered cautiously, since the number of anti-Th/To-positive cases in our study was small (n = 8). Clinically, anti-Th/To is associated with features like puffy fingers, small bowel disease, hypothyroidism, and, less frequently, arthropathy[10]. Previous studies have also demonstrated an association with isolated pulmonary hypertension and pulmonary fibrosis, leading to poor survival[1]. Mitri, *et al* compared the clinical features of anti-Th/To-positive SSc patients with subjects who were ACA-positive[22]. They found that anti-Th/To patients with lcSSc had mild cutaneous, vascular, and gastrointestinal involvement, and they more often had certain features typi-

cally seen in dcSSc, such as pulmonary fibrosis and scleroderma renal crisis, as well as reduced survival compared to the ACA-positive group. The 5- and 10-year cumulative survival rate (84% and 76%, respectively) of ACA-positive patients was significantly better than survival of anti-Th/To patients (76% at 5 years, 58% at 10 years)[1]. Our Italian cohort of anti-Th/To-positive patients was more frequently male and more frequently affected by pericarditis and ILD, although impairment of pulmonary function appeared mild. Moreover, there was no increase in the risk of severe gastrointestinal disease (namely watermelon stomach and angiodysplasia) and renal crisis, and no anti-Th/To-positive patient died during the followup. Thus, our data suggest that Italian lcSSc patients with anti-Th/To do not have worse prognosis, compared with ACA-positive lcSSc patients, despite observation of ILD and pericarditis along with lower FVC.

In summary, our study shows that anti-Th/To antibodies are common among antinucleolar antibody-positive patients with SSc in Italy. Clinically, they are associated with mild cutaneous disease in lcSSc, consistent with previous studies. Anti-Th/To-positive Italian patients with SSc frequently had pericarditis and mild ILD, but they did not develop PAH. Although the relevance of these results may be limited by the relatively low number of anti-Th/To-positive patients identified, the prognosis appears excellent. This is in contrast to previous reports[1,21,23], and whether the difference is due to heterogeneity among Caucasians, environmental factors, or other reasons remains to be determined in future studies. These aspects could also influence the high prevalence of male SSc patients with anti-Th/To antibodies. IIF could help to identify antinucleolar antibody-positive sera, and this calls for further analysis with specific techniques like IP that are not routinely used, and that allow detection of uncommon SSc autoantibodies such as anti-Th/To.

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