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Original article

Autoantibodies to a novel Rpp38 (Th/To) derived B-cell epitope are specific for systemic sclerosis and associate with a distinct clinical phenotype

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Abstract

Objective. Detection of antinuclear antibodies and specific autoantibodies is important in the diagnosis and classification of SSc. Several proteins of the Th/To complex, including Rpp25, Rpp38 and hPop1 are the target of autoantibodies in SSc patients. However, very little is known about the epitope distribution of this autoantigen. Consequently, we screened Rpp25, Rpp38 and hPop1 for B cell epitopes and evaluated their clinical relevance.

Methods. Serum pools with ($n = 2$) and without ($n = 1$) anti-Th/To autoantibodies were generated and used for epitope discovery. Identified biomarker candidate sequences were then utilized to synthesize synthetic, biotinylated, soluble peptides. The peptides were tested to determine reactivity with sera from SSc cohorts ($n = 202$) and controls ($n = 159$) using a chemiluminescence immunoassay. Additionally, samples were also tested for antibodies to full-length recombinant Rpp25 antibodies by chemiluminescence immunoassay.

Results. Several immunodominant regions were found on the three proteins. The strongest reactivity was observed with an Rpp38 peptide (aa 229–243). Autoantibodies to the Rpp38 peptide were detected in 8/149 (5.4%) limited cutaneous SSc patients, but not in any of 159 controls ($P = 0.003$ by two-sided Fisher's exact probability test). Although reactivity to the novel antigenic peptide was correlated with the binding to Rpp25 (rho = 0.44; $P < 0.0001$), subsets of patient sera either reacted strongly with Rpp25 or with the novel Rpp38-derived peptide.

Conclusion. A novel Rpp38 epitope holds promise to increase the sensitivity in the detection of anti-Th/To autoantibodies, thus enhancing the serological diagnosis of SSc.

Key words: systemic sclerosis, autoantibodies, interstitial lung disease, diagnosis, epitope, peptide

CLINICAL SCIENCE

Rheumatology key messages

- Epitope mapping identified several linear epitopes on Th/To antigens.
- The novel Rpp38 (Th/To) derived peptide helps to increase sensitivity.
- Antibodies to the novel Rpp38 (Th/To) B-cell epitope are specific for limited cutaneous SSc.

Introduction

SSc is characterized by the presence of circulating autoantibodies that bind a variety of intracellular antigens, generally referred to as antinuclear antibodies (ANA) [1, 2]. The most prevalent autoantibodies in SSc target topoisomerase I (topo-I, Scl-70), centromere proteins (CENP) and RNA polymerase III, which are also key components of the revised SSc classification criteria [1, 3]. Several other autoantibodies have been reported in SSc sera including autoantibodies targeting the PM/Scl complex (also known as the exosome) [4], U3RNP/fibrillarin [5, 6], U11/12 snRNA [7, 8] and the Th/To autoantigens [9–12].

Anti-Th/To antibodies are typically associated with a homogeneous nucleolar staining by conventional IIF on HEp-2 cells [9, 13], a pattern now designated as AC-08 by the International Consensus on ANA patterns (www.anapatterns.org). In addition to the contribution to accurate diagnosis of SSc, ANA can also be used to stratify SSc patients according to clinically relevant

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phenotypes [14–16]. For example, anti-Th/To antibodies have been associated with the limited cutaneous SSc (lcSSc) subset where the reported prevalence of these autoantibodies ranged from 1% to 13% [9, 17, 18]. In addition to SSc, a few reports have described anti-Th/To antibodies in localized scleroderma, RA and interstitial lung disease (ILD) [19–21].

The Th/To autoantigen is a macromolecular protein-RNA complex (human RNase MRP complex) consisting of at least 10 proteins and catalytic RNA [2, 10]. RNase MRP is a ubiquitously expressed eukaryotic endoribonuclease that cleaves various RNAs, including ribosomal, messenger and mitochondrial RNAs [10]. Almost all protein components of the RNase MRP and the evolutionarily related RNase P complex have been reported as autoantibody targets in patients with ANA-associated rheumatic diseases [10, 11, 20], although Rpp25 [22], Rpp38 [10] and hPop1 [10] have been described as the major target autoantigens. While some studies tested serological cohorts, other investigations analysed selected samples initially identified on the basis of a nucleolar IIF staining pattern [9]. Historically, anti-Th/To antibodies have been mostly detected by immunoprecipitation (IP) [9]. Several years ago, a commercial line immunoassay (LIA) for the detection of anti-Th/To antibodies based on the hPop1 target became available and was evaluated in two independent studies [23, 24]. Just recently, a fully automated chemiluminescent immunoassay (CIA) for the detection of anti-Rpp25 antibodies (research use only) has been developed and evaluated [22, 25]. Lastly, an IP real-time PCR assay has been evaluated [26].

Except for their association with lcSSc, the reported clinical association of anti-Th/To antibodies over the past two decades has been inconsistent. Furthermore, due to technical challenges and limited availability of the IP assay or alternative reliable immunoassays, anti-Th/To autoantibody tests are rarely performed in routine diagnostic algorithms. In addition, very little is known about the B-cell epitope distribution of the major Th/To autoantigens. Consequently, the primary goal of this study was to analyse the epitope distribution on Rpp25, Rpp38 and hPop1 as an approach to adapting the identified epitopes to a high performance immunoassay that may be easily adopted by research and diagnostic laboratories.

Methods

Patient sera

Three pools of sera were generated, each containing equal volumes of three individual samples. The first two pools (Th/To Pool 1 and Th/To Pool 2) were based on samples monospecific for anti-Th/To autoantibodies (anti-Th/To by CIA; reactivity to other SSc-related antigens were excluded using commercial methods) derived from SSc patient sera and a third pool combining three SLE sera (SLE Pool 3) served as negative control pool. Reactivity to Th/To and other autoantigens was measured using CIA (Inova Diagnostics, San Diego, CA). For the evaluation of the assay based on the Rpp38-derived biotinylated peptide, patients fulfilling the classification criteria for SSc [3] were enrolled including 149 with limited cutaneous (lcSSc), 49 with diffuse cutaneous SSc (dcSSc) and four with sine SSc (absence of external skin involvement, but the presence of RP and fibrosis in one or more internal organs) [27].

As controls, samples from patients with SLE ($n = 15$), SS ($n = 5$), idiopathic inflammatory myopathies ($n = 27$), patients with HBV ($n = 20$), HCV ($n = 21$), HIV ($n = 18$), syphilis infection ($n = 20$) and various other conditions ($n = 33$) were included.

Longitudinal samples were available for all anti-Rpp38 antibody positive samples. In addition, 13 patients with primary RP without signs of SSc as well as samples from various disease controls were included ($n = 159$).

Under the terms of this study, all patient information was anonymized prior to analysis, precluding the requirement of written informed consent. All clinical investigation was conducted according to the principles expressed in the Declaration of Helsinki. Biobank and clinical data collection procedures were approved by the CHUM Ethical Review Board.

Recombinant Rpp25 antigen and anti-Rpp25/Rpp38 immunoassay

Recombinant full-length, his-tagged human Rpp25 was generated and purified as previously described and used for CIA [22]. The Rpp25 assay (Inova Diagnostics) is a novel CIA that is currently used for research purposes only and utilizes the BIO-FLASH® instrument (Biokit, Barcelona, Spain), fitted with a luminometer, as well as the hardware and liquid handling accessories necessary to fully automate the assay [28]. For the Rpp25 and Rpp38 screening assays, the two antigens were immobilized on separate beads. Bead mixtures with different ratios of the two beads were generated and a 50 : 50 ratio showed the best discrimination between positive and negative samples.

Epitope mapping using solid phase peptides

The amino acid sequences of the Rpp25 (#NP_060263), Rpp38 (#NP_006405) and hPop1 (#NP_001139333) autoantigens were translated into 15-mer peptides with peptide-to-peptide overlaps of 14 amino acids (aa, Rpp25 and Rpp38) and 13 aa (hPop1) resulting in 1041 different peptides printed in duplicate (2082 peptide spots in all) using solid phase peptide arrays (PEPperPrint, Heidelberg, Germany) [18, 29, 30]. The three serum pools, Th/To Pool 1, Th/To Pool 2 and SLE Pool 3 were used. Epitope mapping was carried out as previously described [29–31].

Synthesis of candidate peptides and detection

Soluble biotinylated peptides were synthesized by FMOC define chemistry as previously reported [32] and purity verified by HPLC. After reconstitution, the peptides were coupled to streptavidin beads and tested by CIA.

IIF on HEp-2 cells

ANA of SSc sera were detected by IIF performed on HEp-2 cells (Antibodies Incorporated, Davis, CA, USA) [21]. Patterns were reported in concordance with the International Consensus on ANA Patterns (ICAP) classification [33].

Statistical evaluation

The data were statistically evaluated using Analyse-it software (Version 3.90; Analyse-it Software, Ltd, Leeds, UK). χ^2, Spearman's correlation and Cohen's kappa agreement test were carried out to analyse agreements between portions and P values <0.05 were considered significant. Descriptive statistics were used to summarize the baseline characteristics of the patients. χ^2, Fisher's exact and Mann–Whitney U tests were used as appropriate and P values <0.05 were considered statistically significant.

Results

Discovery of linear epitopes using high density solid phase peptide array

When the two serum pools containing anti-Th/To autoantibodies and the control SLE pool were used to probe the Rpp25, Rpp38 and hPop1 peptide arrays, the highest intensity signals were found in peptides representing amino acids 226–246 of the Rpp38 antigen (Fig. 1). The identical peptides were identified by both Th/To serum pools, but not by the SLE negative control serum pool. Moreover, the assays identified some additional but weaker epitopes of Rpp38 and hPop1 (Fig. 1).

Next, all peptides that yielded at least 200 units of reactivity (at 1 : 250 serum dilution) with at least one of the Th/To serum pools, but not with the SLE pool, were selected for further analyses. For Rpp25, one peptide was positive for both pools, 10 peptides for Th/To pool 1 and 7 peptides for Th/To pool 2. Rpp38 displayed a greater number of reactive peptides. Seven peptides were positive for both pools, six peptides for Th/To pool 1 and 24 peptides for Th/To pool 2. Last, two hPop1 peptides were positive for both Th/To pools, 16 peptides for Th/To pool 1 and three peptides for Th/To pool 2. The number and percent of positive peptides (>200 units) and highest signals are summarized in Fig. 2.

Verification of identified epitopes using synthetic Th/to derived soluble peptides

A total of eight soluble biotinylated peptides were designed based on the identified sequences and used for further studies (Table 1). Two peptides were based on the hPop1 sequence, three on Rpp25, two on Rpp38 and one was a Rpp38 and hPop1 hybrid (see Table 1). In further analysis, peptide Rpp38- ^{229} RELLDTSFEDLSKPK ^{243}, Rpp38- ^{233} DTSFEDLSKPKRKLA ^{247} and Rpp38- ^{233}DTSFEDLSKPKRKLA ^{247}/hPop1- ^{418} TGIIISDLTMEMNRF ^{432} were most reactive. Further comparative descriptive analysis showed significantly higher reactivity against peptide Rpp38- $^{229–243}$ and Rpp38- $^{233–247}$ with the SSc sera. The best discrimination between SSc and controls was observed with Rpp38- ^{229} RELLDTSFEDLSKPK ^{243}, which was then used for further analysis in a clinical study.

Prevalence and titres of anti-Rpp25 and Rpp38 antibodies in different diseases

When the prevalence and the titres of anti-Rpp25 and anti-Rpp38 peptide antibodies were studied in SSc and various other diseases, the highest prevalence and titres were found with SSc sera (Fig. 3). Anti-Rpp25 antibodies showed a higher sensitivity, but lower specificity compared with anti-Rpp38 antibodies. Strikingly, the reactivity to the Rpp38 derived peptide was very specific for SSc and restricted to the limited cutaneous form.

Correlation between anti-Rpp25 and anti-Rpp38 peptide antibodies and development of anti-Th/to screening assay

Antibodies to the novel Rpp38 peptide showed significant correlation with antibodies to full length human recombinant Rpp25 as measured by CIA. Using 299 serum samples (202 SSc, 97 controls), the quantitative agreement according to the Spearman equation was (rho = 0.44, 95% CI 0.35, 0.53; $P < 0.0001$). However, there were subsets of SSc sera that either reacted strongly with Rpp25 or with the novel Rpp38 derived peptide (see Fig. 4). When beads coupled with Rpp25 were mixed with Rpp38 coupled beads in a 50 : 50 ratio and tested with four samples positive for Rpp25 but negative for Rpp38 and two samples positive for Rpp38 but negative for Rpp25, it was found that the bead mixture detected both autoantibody specificities. Negative samples remained negative and double positive samples remained positive.

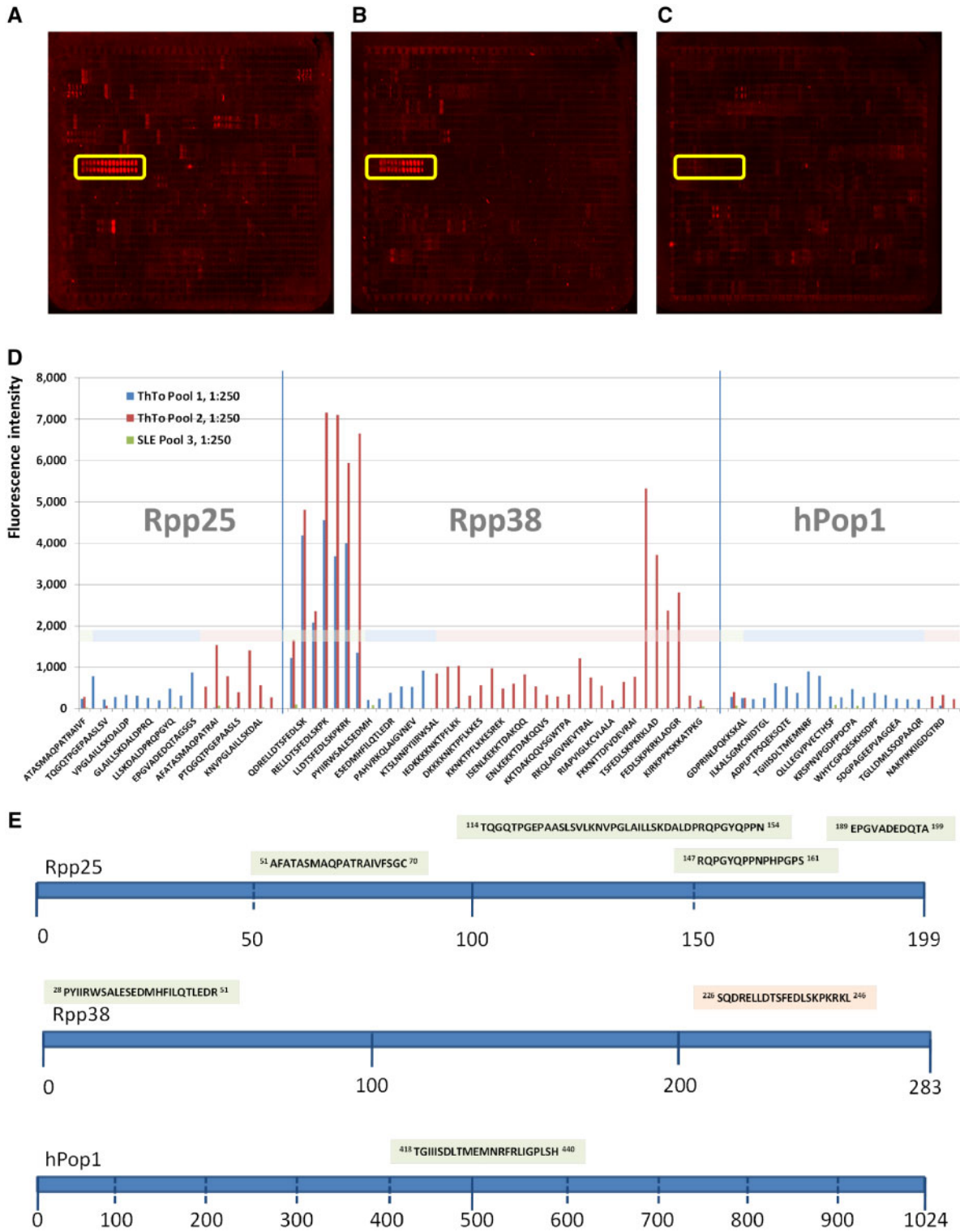
Sequence comparisons between Rpp25 and Rpp38

Due to the observed correlation between the reactivity of autoantibodies to Rpp25 and the Rpp38 derived peptide, a sequence alignment was carried out that showed limited similarity/identity between the two molecules (Supplementary Fig. S1, available at *Rheumatology* online). A total of four regions were identified on both proteins that showed >30% sequence identity, none of them overlap with the identified major Rpp38 epitope.

Clinical features of anti-Rpp38 peptide antibody positive patients

We identified eight (seven females and one male) patients that tested positive for anti-Rpp38 peptide antibodies in at least one serum sample and for which clinical data could be retrieved from medical records. Seven had lcSSc and one had early lcSSc. The median age at disease onset was 34.6 years (7.8–48.7 years) and the median age at blood sampling was 51.0 years (32.2–73.9 years). Of the eight patients, two had co-morbid pulmonary fibrosis, two had SS and two had primary biliary cholangitis. In six of the eight patients, anti-Rpp38 antibodies were detectable in the first longitudinal serum sample. The remaining two patients developed anti-Th/To antibodies over the course of their disease. Although anti-Rpp38

FIG. 1 Epitope mapping of Rpp25, Rpp38 and hPop1 using high density peptide arrays



The peptide arrays stained with pools of Th/To positive SSc serum samples (**A**, **B**) show several immunoreactive regions in all proteins. Reactivity with the pool of samples derived from SLE sera showed minor reactivity (**C**). Peptides that showed reactivity (>200 units) with at least one of the two Th/To serum pools, but not with the SLE pool are shown in **D**. In **E**, epitope map highlights main reactive peptides.

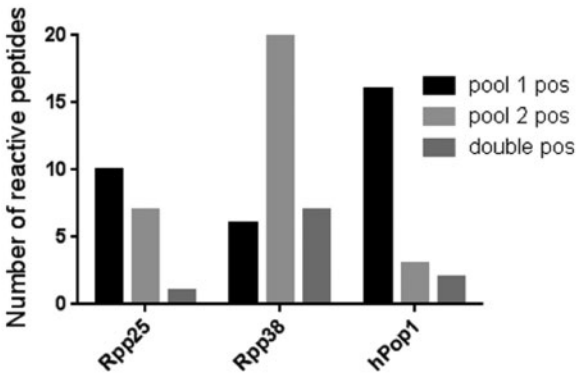
antibodies were exclusively found in patients with lcSSc (8/149 lcSSc *vs* 0/49 dcSSc; OR 6.0, 95% CI 0.33, 104.94), the difference between the two clinical subsets did not reach statistical significance ($P = 0.2235$).

Nevertheless, anti-Rpp38 antibodies were significantly more common in patients with lcSSc than in all other controls including the dcSSc (8/149 *vs* 0/225, $P = 0.0006$ by two-sided Fisher's exact probability test, OR 27.1, 95% CI 1.6, 473.04). Of the eight patients with anti-Th/To antibodies, five had other antibodies in addition to anti-Th/To, with anti-Ro52 being the most common one followed by anti-centromere and anti-PM/Scl (see Table 2).

Association of anti-Rpp38 antibodies with IIF pattern

Of the eight anti-Rpp38 peptide positive samples, three showed a homogeneous nucleolar pattern (ICAP AC-08) as the main pattern. In the remaining five samples, AC-08 was the secondary IIF pattern. Within the group of dcSSc patients, 12/49 (24.5%) showed a nucleolar pattern, but all were negative for anti-Rpp38 antibodies confirming the association with lcSSc.

Fig. 2 Number of linear peptides on Rpp25, Rpp38 and hPop1 identified by Th/To pools



The number of peptides that showed a reactivity of >200 units with at least one of the Th/To pools, but not with the SLE control pool, are displayed. For both Rpp25 and hPop1, more peptides reacted with pool 1, but not with pool 2. Rpp38 showed the highest number of peptides that reacted with both Th/To sample pools.

Discussion

This is the first study to describe linear epitopes on three Th/To antigens. In addition, we developed a particle-based assay using the newly identified Rpp38 epitope that showed high specificity for lcSSc and identified patients with a distinct clinical phenotype.

ANA are detected in >90% of SSc patients' sera and are valuable tools in establishing the diagnosis, predicting the onset, internal organ involvement and the prognosis of the disease [1, 34]. Besides anti-centromere, anti-topo I/Scl-70 and anti-RNA polymerase III antibodies, which are part of the classification criteria of SSc, autoantibodies to the Th/To complex have also been described in up to 10% of SSc sera [9–12, 18]. Almost all protein components of the Th/To complex have been reported to be targets of the anti-Th/To autoantibody repertoire [10, 20]. Moreover, recent studies using ELISA and CIA confirmed Rpp25 as the major Th/To autoantigen, being detected in ~60–100% of anti-Th/To positive patients (as identified by IP) [10, 22].

The observation that they are primarily detectable in SSc makes this specificity an important serological adjunct in the diagnosis and clinical stratification of SSc. In addition, a high prevalence (~25%) of anti-Th/To antibodies was found in ANA-positive/ENA-negative SSc patients [25]. In our study, all samples with anti-Rpp38 antibodies showed the AC-08 (nucleolar) pattern, which is consistent with the presence of anti-Th/To antibodies [35]. The anti-Th/To antibody test may also have applications for non-SSc patients such as those with ILD, as anti-Th/To antibodies were reported in ~50% of anti-nucleolar antibody-positive idiopathic pulmonary fibrosis patients [13]. However, this requires further studies on large idiopathic pulmonary fibrosis cohorts as only a portion of these patients expressed nucleolar pattern.

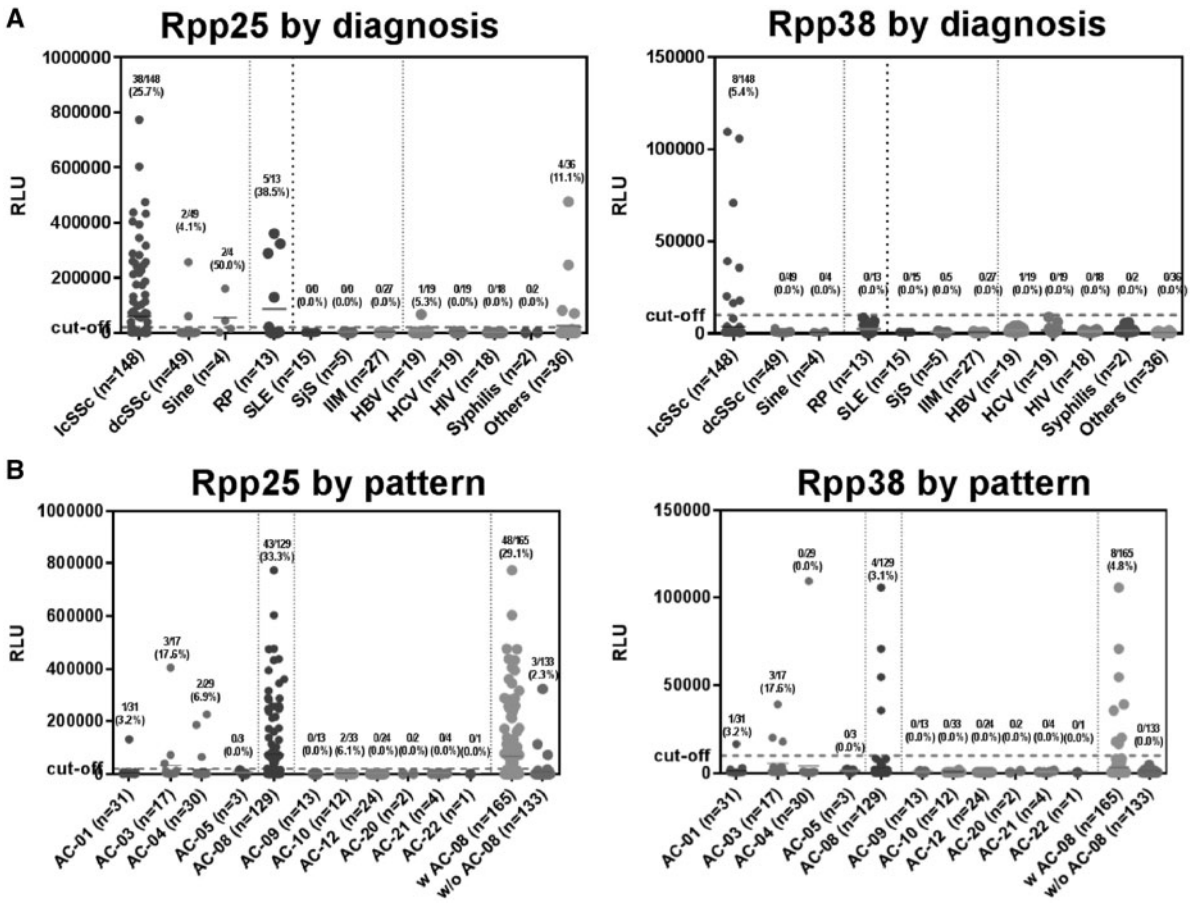
When comparing the prevalence of anti-hPop1 or anti-Rpp25 antibodies in SSc patients based on published studies, similar prevalences were found (2.1–3.3%) [22–24]. However, statistically significant differences in the clinical specificities were reported 98.7% [23] and

Table 1 Designed Rpp25, Rpp38 and hPop1 peptides

No.	Antigen	aa	Short ID	ID
1	hPop	15	^{400} hPop1 ^{414}	hPop- ^{400} IGDGTRDPCLPYSWI ^{414}
2	Rpp25	20	^{51} Rpp25 ^{68}	Rpp25- ^{51} AFATASMAQPATRAIVFSGC ^{68}
3	Rpp38	15	^{229} Rpp38 ^{243}	Rpp38- ^{229} RELLDTSFEDLSKPK ^{243}
4	Rpp25	30	^{54} Rpp25 ^{68} / ^{123} Rpp25 ^{137}	Rpp25- ^{54} TASMAQPATRAIVFS^{68} / Rpp25- ^{123} AASLSVLKNVPGLAI ^{137}
5	Rpp25	26	^{189} Rpp25 ^{199} / ^{123} Rpp25 ^{137}	Rpp25- ^{189} EPGVADEDQTA^{199} / Rpp25- ^{123} AASLSVLKNVPGLAI ^{137}
6	Rpp38	15	^{223} Rpp38 ^{247}	Rpp38- ^{233} DTSFEDLSKPKRKLA ^{247}
7	hPop1	23	^{418} hPop1 ^{440}	hPop1- ^{418} TGIIISDLTMEMNRFRLIGPLSH ^{440}
8	Rpp38/hPop1	30	^{233} Rpp38 ^{247} / - ^{418} hPop1 ^{432}	Rpp38- ^{233} DTSFEDLSKPKRKLA ^{247} / hPop1- ^{418}TGIIISDLTMEMNRF ^{432}

aa: amino acid; ID: identification code.

FIG. 3 Autoantibody reactivity to Rpp25 and to the major Rpp38 peptide epitope

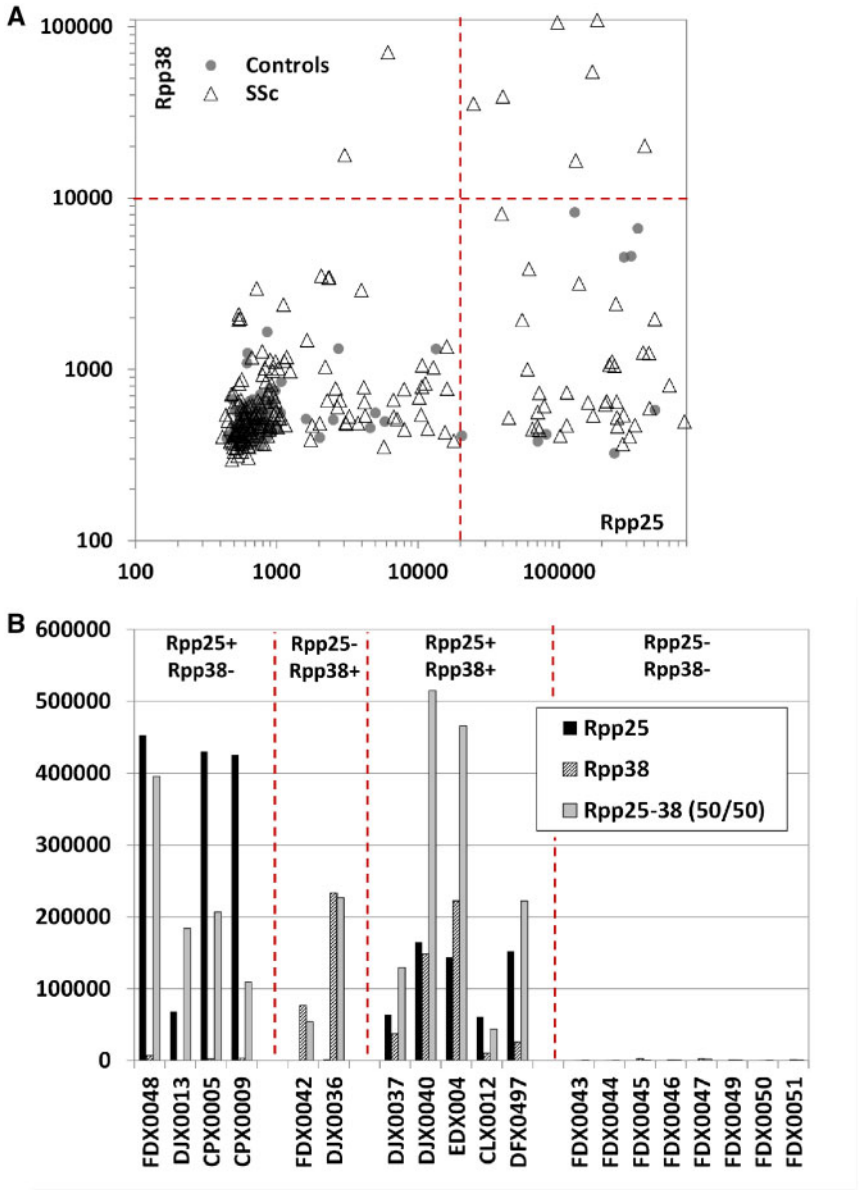


The levels of anti-Rpp25 (recombinant full-length protein) and anti-Rpp38 (the major Rpp38 peptide) antibodies are shown as measured by chemiluminescence immunoassay (CIA) in **A**. In **B**, reactivity is displayed according to the IIF pattern. The individual ICAP pattern as well as two groups [with (w) and without (w/o) any AC-08 pattern] are presented. lcSSc: limited cutaneous SSc; dcSSc: diffuse cutaneous); IIM: idiopathic inflammatory myopathies; HBV: hepatitis B virus; HCV: hepatitis C virus; HIV: human immunodeficiency virus; AC-01: homogeneous; AC-03: centromere; AC-04: fine speckled; AC-05: large coarse speckled; AC-08: homogeneous nucleolar; AC-09: clumpy nucleolar; AC-10: punctate nucleolar; AC-12: punctate nuclear envelop; AC-20: cytoplasmic fine speckled; AC-21: cytoplasmic anti-mitochondrial; AC-22: cytoplasmic Golgi-like.

97.8% [24] for hPop1 by LIA *vs* 99.5% for Rpp25 by CIA [22]. Whether the differences are attributable to different control groups is unknown and should be analysed in future studies.

In a recent study on a large cohort of Canadian SSc patients, the LIA used to detect anti-hPop1 missed a significant number ($n = 18$) of anti-Th/To antibody positive samples that were identified by IP [25]. This may be due to low prevalence of anti-hPop1 autoantibodies among anti-Th/To positive patients in this cohort or low sensitivity of the hPop1 antigen used in LIA. However, 20% of the anti-Th/To antibodies positive samples were also missed using the Rpp25 CIA when compared with IP. An analysis of the lack of concordance and the potential complementarity of anti-Rpp25 and anti-hPop1 antibodies is currently ongoing. In a study by Kuwana *et al.* [20], anti-hPop1 autoantibodies were significantly more prevalent in anti-Th/To positive SSc patients as detected by IP, compared with anti-Th/To positive patients with other AARDs. In contrast, Rpp30 and Rpp38 were equally targeted by autoantibodies from SSc and non-SSc ANA-associated rheumatic disease patients. This is inconsistent with our findings, which showed that reactivity of an SLE serum pool to linear peptides of hPop1 was higher compared to Rpp25 and Rpp38. However, discontinuous conformational epitopes were not analysed in our study. During the course of this study, we also aimed to compare the reactivity to the individual Th/To components using a recombinant Rpp38 and hPop1. However, we were unable to develop an immunoassay or functional assays that might be related to the recombinant constructs. Nevertheless, when indirectly comparing the reactivity to

FIG. 4 Autoantibodies to Rpp25 and Rpp38



A: Correlation between anti-Rpp25 and anti-Rpp38 $^{229-243}$ peptide antibodies. The reactivity between anti-Rpp25 and anti-Rpp38 peptide antibodies measured in 299 samples (203 SSc, 81 controls and 13 with unknown diagnosis) using a chemiluminescence assay showed significant correlation (rho = 0.44, 95% CI 0.35, 0.53; $P < 0.0001$). However, subsets of some patients either reacted with Rpp25 or with the novel Rpp38 derived peptide. **B**: Using a mixture of Rpp25 and Rpp38 coupled beads, samples that were positive for either of the two antigens were detected. All values on the ordinate axis are expressed as relative light units (RLU). QF: QUANTA Flash.

Rpp38 described in the original study (6/303, 2%), the identified Rpp38 epitope showed comparable reactivity.

Although autoantibodies to Th/To have been known for over two decades, the clinical associations of anti-Th/To antibodies are not yet fully established. Previous studies are mostly consistent in showing an association with lcSSc; however, association with more specific clinical features is somewhat inconsistent. Small numbers of anti-Th/To positive patients, differences in ethnicity and environment, differences in the detection methods, recruitment bias and other factors could explain the inconsistencies [9, 11, 36–38]. Anti-Th/To antibodies have also been associated with pericarditis, ILD and a high frequency of isolated pulmonary hypertension [9, 17]. Compared with anti-CENP IIF positive patients, anti-Th/To lcSSc patients have milder cutaneous, vascular and

Table 2 Clinical features of SSc patients with anti-Rpp38 antibodies

#	Sex	Diagnosis	Other	Age at onset	Age at sampling	ILD at blood sampling	ILD during disease	Pulmonary hypertension	Capillaroscopy	Other aab
1	F	lcSSc	PBC	7.8	53.1	0	0	0	1-Late pattern	Ro52, CENP
2	F	lcSSc	PBC	34.6	48.8	0	0	0	1-Late pattern	Ro52, PM/Scl
3	M	lcSSc		23.3	36.6	1	1	0	1-Late pattern	none
4	F	lcSSc	C, DU	43.1	73.9	0	0	0	1-Late pattern	Ro52
5	F	lcSSc	DU	NA	36.1	0	1	0	NA	Ro52, CENP, PM/Scl
6	F	early SSc	SS	30.5	32.2	0	1	0	1-Early pattern	CENP
7	F	lcSSc	SS	45.6	73.9	0	1	0	1-Late pattern	none
8	F	lcSSc	none	48.7	57.7	0	0	0	1-Late pattern	none

lcSSc: limited cutaneous SSc; C: calcinosis; CENP: centromere protein; DU: digital ulcers; NA: not available; PBC: primary biliary cholangitis; f: female; m: male; 0: absent; 1: present.

gastrointestinal involvement, but more often have pulmonary fibrosis, renal crisis and reduced survival [36]. Like other SSc-related autoantibodies, the presence of anti-Th/To antibodies in patients with isolated RP is predictive of early SSc [39]. Also, anti-Th/To positive SSc patients demonstrated earlier development of nailfold capillary abnormalities than anti-CENP B positive patients [39]. In addition, anti-Th/To positive patients were younger and more frequently male compared with anti-CENP IIF-positive patients [9]. The prevalence of anti-Th/To antibodies might be higher in Caucasian Americans compared with African and Latin American patients [37]. Lastly, in the Canadian SSc cohort preselected for autoantibody reactivity, the association of anti-Th/To and anti-Rpp25 antibodies with ILD and nailfold capillary abnormalities was confirmed [25]. In light of the importance of stratifying SSc patients in more clinically meaningful subsets of patients [14, 38], it is interesting to note that all eight anti-Rpp38 antibody positive subjects had lcSSc and four patients developed ILD during disease cause. This potential association is of interest as anti-Th/To antibodies fluctuate over time and might predict future development of ILD [40]. However, because the prevalence of ILD in the anti-Rpp38 positive individuals is not significantly different from general SSc cohorts [41], future research might also consider severity of ILD and prognostic aspects. Although not statistically significant, it is intriguing that two of the eight (25%) patients had primary biliary cholangitis (PBC), an uncommon autoimmune liver condition that may coexist with lcSSc [42]. This observation deserves further investigation.

Several studies have analysed the epitope distribution on SSc-related autoantigens, including CENP, PM/Scl [43], RNA Pol III and topo I [1]. For CENP-A and PM/Scl, linear epitopes could be identified using the SPOT technology and these epitopes were then used to develop sensitive and specific ELISA [44, 45]. In contrast, very little has been published about the epitope specificity and distribution on Th/To autoantigens. This is the first study to describe linear epitopes on the Rpp25, Rpp38 and hPop Th/To autoantigens. The identified major epitope on Rpp38 was then used to develop an immunoassay showing significant correlation to the results obtained with the anti-Rpp25 immunoassay, which raised questions about potential cross-reactivity. A sequence comparison between Rpp25 and Rpp38 revealed low sequence similarity. In addition, the major Rpp38 epitope does not show sequence similarity to any region on Rpp25. Consequently, the correlation might be explained by a polyclonal autoantibody response to several members of the protein complex rather than by cross-reactivity. In the future, studies using, for example, the entire Canadian Scleroderma Research Group (CSRG) SSc cohort, or patients registered at the EULAR Scleroderma Trials and Research (EUSTAR) [18, 46] or the German Network for systemic scleroderma [18] and/or the Australian SSc cohort [47] would be of great interest to thoroughly analyse the clinical associations of autoantibodies to the new Th/To epitopes. This could also provide further insights about the future use of Rpp25 and Rpp38 peptide, either as single analytes, or as a screening approach to assess anti-Th/To antibodies as demonstrated herein.

Despite the relatively low prevalence of anti-Th/To antibodies in SSc, testing for these antibodies and the sub-specificities (anti-Rpp25, anti-Rpp38 and anti-hPop1 antibodies) may have significant value for patient stratification [5, 14]. The association with ILD is of particular importance because lung disease accounts for 33% of SSc-related deaths [48].

In summary, this is the first study to describe linear epitopes on the Th/To antigen. In addition, the assay based on the identified epitope was specific for lcSSc and identified patients with a distinct clinical phenotype.

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Supplementary data

Supplementary data are available at *Rheumatology* online.

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