Presence of anti-eIF2B, anti-RuvBL1/2 and anti-synthetase antibodies in patients with anti-nuclear antibody negative systemic sclerosis

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Abstract

Objectives: Autoantibodies targeting ubiquitously expressed nuclear antigens can be identified in most patients with systemic sclerosis (SSc). Cytoplasmic autoantibodies (in otherwise anti-nuclear antibody (ANA)-negative sera) targeting eukaryotic Initiation Factor-2B (anti-eIF2B) have recently been identified in SSc with clinical associations to diffuse cutaneous disease (dcSSc) and interstitial lung disease (ILD); although the majority of samples originated from a tertiary SSc-ILD centre. We investigated the prevalence and clinical associations of recently described SSc-specific (including anti-eIF2B) and other cytoplasmic autoantibodies in ANA-negative sera obtained from a large representative SSc cohort.

Methods: ANA-negative sera from the Scleroderma Family Registry and DNA Repository underwent indirect immunofluorescence, radiolabeled protein immunoprecipitation (IPP) (+/- immunodepletion) to identify anti-eIF2B and other connective tissue disease-related autoantibodies. The clinical phenotype of positive samples was evaluated.

Results: IPP was performed on 128 ANA-negative samples (obtained from 3249 SSc patients). Anti-eIF2B antibodies were present in 9 samples (7%); the majority of whom had dcSSc (8/9). SSc-ILD was present in all anti-eIF2B patients for whom chest imaging was available (7/9). Anti-synthetase autoantibodies (targeting PL12, PL7, OJ and Zo) were identified in 7 samples (5.5%); all of whom fulfilled the 2013 ACR/EULAR classification criteria for SSc and had evidence of SSc-ILD where relevant outcomes were available for evaluation. Anti-RuvBL1/2 antibodies were identified in 2 patients with SSc-overlap syndromes.

Conclusions: Anti-eIF2B antibodies are cytoplasmic SSc-specific autoantibodies with strong clinical associations with dcSSc and SSc-ILD found in ANA-negative sera. Anti-synthetase autoantibodies, and other recently discovered SSc-specific antibodies such as anti-RuvBL1/2, can also be identified in ANA-negative SSc.

Abstract word count: 250

Introduction

Identification of autoantibodies targeting intracellular antigens helps support diagnosis and predict disease course in systemic sclerosis (SSc) (1). The majority of SSc-specific and SSc-associated autoantibodies target nuclear proteins and their presence is sometimes suggested by characteristic anti-nuclear antibody (ANA) staining patterns on indirect immunofluorescence (IIF). Commercially available addressable laser bead immunoassays (ALBIA), enzyme-linked immunosorbent assay (ELISA) and line immunoassays (LIA) identify the antigen target for the majority of ANA-positive SSc samples following IIF. Radiolabelled protein immunoprecipitation (IPP) is the gold standard for confirming the presence of autoantibodies to intracellular antigens in SSc. Previous registry analysis has detected ANA on IIF in 98.3% of patients with SSc, within whom SSc-specific or SSc-associated autoantibodies were characterised in 92.2% using commercially available methods (ALBIA, ELISA and LIA), rising to 96.5% following IPP (2).

Antibodies targeting eukaryotic Initiation Factor 2B (anti-eIF2B) were recently identified (using IPP) in seven patients with SSc; each of whom were negative for existing SSc-specific autoantibodies and had a fine cytoplasmic speckle (FCS) on IIF but no direct nuclear staining (i.e. “ANA negative”) (3). Anti-eIF2B antibodies were associated with diffuse cutaneous SSc (dcSSc) and interstitial lung disease (ILD); although selection bias was likely as the majority of samples were obtained from a large tertiary SSc-ILD centre. The principal objectives of the present study was to use IPP to investigate the prevalence and clinical associations of anti-eIF2B antibodies (and additional CTD-specific autoantibodies) in “ANA-negative” samples obtained from a larger, more representative cohort of SSc (4).

Methods

Study population

Samples were obtained from the Scleroderma Family Registry and DNA Repository that has enrolled patients from 11 US scleroderma centres and participating Canadian Scleroderma Research Group

sites as previously described (4, 5). IRB approval was obtained at all participating sites and all participants provided informed written consent (4, 5). The study pre-dated the 2013 ACR/EULAR classification criteria for SSc but all patients fulfilled the 1980 ARA preliminary classification criteria for SSc and/or had ≥3 of the 5 clinical features of the CREST syndrome (Calcinosis, Raynaud's phenomenon, (o)Esophageal dysfunction, Sclerodactyly or Telangiectasias) with sclerodactyly being mandatory (6, 7), ensuring a representative scleroderma patient cohort.

ANA-negative samples

To be considered ANA-negative in the earlier study (4), IIF was negative on HEp-2 cells (titre 1:80, assessed by a single investigator), passive immunodiffusion (ID) using commercial assays for anti-Scl70, anti-Ro60, anti-La (Inova Diagnostics, San Diego, USA) and ELISA for anti-RNA polymerase III (MBL, Co. Ltd, Japan).

Laboratory investigations

ANA-negative samples (with sufficient available serum) underwent repeat IIF using HEp-2 cells and fluorescein-labelled anti-Human IgG (Inova Diagnostics, San Diego, USA), with patterns assessed at titre 1:40 by a single investigator (HL) followed by IPP as previously described (3)..

Sera that immunoprecipitated a 30kDa band underwent immunodepletion to confirm antigen specificity for eIF2B as previously described (3).

Sera that immunoprecipitated a doublet (molecular weights ~50kDa) were analysed for suspected anti-RuvBL1/2 antibodies. IPP was completed using either 40 µl sera or 20 µl polyclonal rabbit anti-RuvBL1, 2 mg protein-A-Sepharose and 5 mM Suberic-acid-bis-sodium. Beads were incubated with 1 ml K562 extract (prepared from 28 x 10^6 cells/ml) for 3 hr and re-suspended in Sample Buffer. Immunoprecipitates were fractionated by 10% SDS-PAGE, transferred to nitrocellulose and probed

with anti-RuvBL1 (1:500 dilution) for 90 min. Bands were detected using an alkaline-phosphatase conjugated goat anti-rabbit IgG (1:0000 dilution) and BCIP/NBT substrate solution (all chemicals obtained from Sigma, UK).

Assessment of clinical associations

Definitions of disease-associated clinical features for patients enrolled in the Scleroderma Family Registry and DNA Repository has been described previously (4). In brief, disease subtype (limited cutaneous [lcSSc] versus dcSSc) was based on the physician-assessed extent of skin involvement at enrolment (7). There were no study-mandated procedures and all investigationswere requested at the discretion of the treating physician. SSc-ILD was defined as the presence of honeycombing, increased interstitial markings or ground glass opacity on either chest radiograph, chest computed tomography (CT) and/or the presence of restrictive lung disease indicative of ILD determined by a forced vital capacity (FVC) of <70% predicted. Within this cohort, pulmonary function test results were available for analysis in 80.8% of cases and chest imaging using radiograph, standard CT, or high-resolution CT (HRCT) was available in 23.3%, 12.3% and 23.2% of patients respectively. Arthropathy was defined as documented synovitis and/or joint pain.

Results

Of 3249 patients enrolled in the study, 208 (6.4%) were initially considered “ANA negative”. Sufficient serum was available for 128 ANA-negative samples (Figure 1A). Repeat IIF remained negative for nuclear staining in 46 samples (36%), although 25 of these samples exhibited a cytoplasmic speckle on IIF (6 coarse and 19 FCS), with IPP bands present in 21/25 subjects. Seven subjects with a FCS exhibiting a 30kDA band on IPP were subsequently confirmed as anti-eIF2B antibodies on immunodepletion (Figure 1B) and a further 7 subjects with a FCS were positive for antibodies targeting tRNA synthetases including anti-PL12 (n=3), anti-PL7 (n=2), anti-OJ (n=1) and anti-Zo (n=1) antibodies (Figure 1C). The remaining 7 subjects exhibited unknown bands (no apparent commonality). The

remaining 4 “ANA negative” subjects with a cytoplasmic speckle on repeat testing had no visible bands on IPP.

Within the 21 patients that remained “ANA negative” on repeat IIF *without* a cytoplasmic speckle, IPP identified one subject with anti-Ro antibodies and another subject with anti-RuvBL1/2 antibodies (Table 1, Figure 1C).

A combination of nuclear staining patterns were present in the remaining 82 samples (16 nucleolar, 10 homogeneous, 26 fine speckle and 30 coarse speckle). Within this group, IPP identified established SSc-specific and SSc-associated autoantibodies in 21 samples (16.4%), including anti-RNA Pol I/III (n=9, two of which also exhibited anti-Ro), anti-U1RNP (n=3), anti-topoisomerase (n=1), anti-U3-RNP (n=1), and anti-Ro (n=4, one of which contained anti-La and one with concomitant anti-mitochondrial antibodies). Two additional samples were positive for anti-eIF2B antibodies (each of which had a fine speckled nucleolar sparing (FSNS) nuclear-staining pattern alongside a FCS). Anti-RuvBL1/2 antibodies were identified in a sample with a FSNS nuclear staining pattern on IIF. Of the remaining 61 patients with anti-nuclear staining on repeat IIF, unknown bands were identified in 30 subjects (no commonality), whereas 31 subjects had no visible IPP bands.

Across the sample cohort (n=128), antibodies with a known antigen target were present in 37 subjects (28.9%), unknown bands in isolation were identified in 46 subjects (35.9%), rising to 73 (57%) when unknown bands were identified in conjunction with a known antibody. No visible bands were identified on IPP in 45 samples (35.2%).

The IIF pattern and clinical features of the 9 anti-eIF2B positive patients are summarized in Table 1. The majority of patients had dcSSc (8/9, 89%), although the mean modified Rodnan Skin Score (documented in 7/9) was only 17.7units. All patients with previous chest imaging (7/9, 78%) had radiographic evidence

of SSc-ILD; either on computed tomography (6/6, 100%) and/or plain radiograph (4/4, 100%). Pulmonary function tests (documented in 7/9, 78%) revealed a mean FVC of 63.4% predicted and mean DLco of 64.3% predicted. The prevalence of digital ulcers (2/9) and pulmonary arterial hypertension (PAH) (1/9) was low. Joint involvement was common (6/7 were documented) and 2 patients with anti-eIF2B antibodies were considered to have an overlap syndrome with rheumatoid arthritis (RA). Despite the major association with myositis-spectrum disorders (MSD), all 7 patients with positive anti-synthetase antibodies fulfilled 2013 ACR/EULAR classification criteria for SSc (8) and only one patient had documented evidence of myopathy (not considered part of a polymyositis overlap). One patient with anti-RuvBL1/2 had dcSSc complicated overlap features of polymyositis, whereas the second had lcSSc with overlap features of Sjogren's syndrome (Table 1).

Discussion

This is the largest study of "ANA-negative" SSc to incorporate IPP undertaken to date. We have confirmed the previously reported clinical association of anti-eIF2B autoantibodies with dcSSc and SSc-ILD in a larger, more representative SSc patient population. Our earlier study suggested a high prevalence of overlap syndrome in anti-eIF2B positive patients with SSc but only 2/9 were classified as such in this study (both RA). Previous work suggests anti-eIF2B antibodies are specific to SSc (not found in healthy controls or patients with other forms of autoimmune rheumatic disease) and are not found in SSc patients with positive nuclear staining on IIF (3). Our findings suggest ~75% (14/19) of SSc patients with a negative anti-nuclear stain but a positive FCS pattern on IIF carry either anti-eIF2B antibodies or anti-synthetase antibodies (but never in combination).

Autoantibodies targeting cytoplasmic tRNA synthetases have previously been described in SSc (2, 9) highlighting the considerable overlap in clinical phenotype that exists between the SSc and myositis-spectrum disorders. Anti-synthetase antibody positive patients in this study fulfilled contemporary

classification criteria for SSc (8), challenging the validity of the term “myositis-specific autoantibodies” to describe anti-synthetase antibodies. None of the anti-synthetase antibody positive patients had been formally classified as overlap syndrome with polymyositis (or other rheumatic diseases), although relevant clinical features such as “mechanics hands” were not routinely captured in this study of SSc. A myopathy was recorded in the patient with anti-OJ (Table).

This is the second study to report the clinical features of anti-RuvBL1/2 antibodies in SSc and our findings strengthen the previously reported association with SSc-overlap syndromes (present in ~60% of original case series) (10). RuvBL1/2 is an important modulator of transcriptional activation, protein assembly (including certain RNPs and RNA polymerase) and essential for cell proliferation (11). It is mainly localized in the nucleus but can be present in the cytoplasm. Anti-RuvBL1/2 antibodies have also been reported in a case of polymyositis with morphoea (12). This study was not appropriately designed to evaluate the prevalence of anti-eIF2B and anti-RuvBL1/2 autoantibodies in SSc but previous estimates have suggested rates of 1.8% and 1.7% respectively (3, 10).

The identification of anti-nuclear staining patterns on repeat HEp2 IIF testing is not unexpected, in view of differences in screening titre, the use of non-standardised reagents between laboratories andsubjective thresholds applied to reporting staining patterns. We deliberately applied a low threshold for reporting IIF staining patterns to maximize the sensitivity for detecting antibodies within this sample cohort. Established SSc-specific and SSc-associated autoantibodies (excluding the more recently described anti-eIF2B and anti-RuvBL1/2 antibodies) were present in 14% of this previously designated “ANA-negative” cohort, that had not been identified on earlier screening investigations including immunodiffusion and ELISA. Some antibody specificities e.g. anti-U3-RNP are not routinely identified using these techniques but others e.g. anti-RNA Pol III are normally captured. Identification of autoantibodies using IPP in sera previously negative to alternative immunoassays capable of identifying such specificities has previously been demonstrated and highlights the variable sensitivity of different methods of antibody detection compared to the gold standard of IPP [2, 9]. The emergence and

refinement of relatively inexpensive commercially available line immunoassays for SSc-specific and myositis specific autoantibodies will improve the sensitivity of autoantibody assessment in future studies, potentially reducing reliance on more specialist techniques such as IPP. Unknown bands on IPP are not uncommon and our preliminary analysis for commonality of the bands has not yet identified any novel antigen targets in SSc using these samples.

Direct comparison between the significance of “ANA negative” SSc in the present study and previous registry analyses is challenging due to differences in disease classification, definitions surrounding “ANA negativity” and immunoassay methods applied. In the present study, the absence of nuclear/cytoplasmic staining on IIF and the absence of detectable known autoantibodies using IPP (including newer antibodies such as anti-eIF2B) occurred in only 19 samples (from 3249 patients, 0.58%). This is lower than an estimate of 1.7% of SSc patients being “autoantibody negative” in an earlier smaller study applying similar investigative techniques [2]. The term “autoantibody negative” in this context might be misleading as the investigative techniques cannot fully exclude the presence of antibodies targeting cellular (e.g. anti-endothelial cell) or cell-surface (e.g. anti-PDGF receptor) antigens. Our findings indicate that SSc is very rare in patients without nuclear/cytoplasmic staining on IIF, in whom a detectable antibody specificity using IPP cannot be identified. This study also highlights the value of an extended array of investigative techniques for autoantibody detection when evaluating SSc-spectrum disorders (particularly should a FCS is observed on IIF in the absence of nuclear staining).

Key Messages:

- Anti-eIF2B antibodies associated with dcSSc and ILD and have a fine cytoplasmic speckle on IIF
- Anti-synthetase antibodies occur in patients fulfilling classification criteria for SSc without features of myositis-spectrum disorders
- Only 0.6% of SSc patients are “autoantibody negative” following IIF and IPP studies

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Legends for table and figure

Table 1. Clinical features of patients positive for anti-eIF2B, anti-synthetase antibodies and anti-RuvBL 1/2 antibodies

ANA, antinuclear antibody; IIF, indirect immunofluorescence; ACR, American College of Rheumatology; ILD, Interstitial Lung Disease; SRC, Scleroderma Renal Crisis; MAL, Malabsorption; RP, Raynaud's Phenomenon; GORD, Gastro-Oesophageal Reflux Disease; CAL, Calcinosis; TEL, Telangiectasia; DU/DP, Digital ulceration/Digital pitting; PAH, Pulmonary Artery Hypertension; Neg, negative; FCS, fine cytoplasmic speckle; dcSSc, diffuse cutaneous systemic sclerosis; N/A, not available; FS NS, fine speckle nucleolar sparing; M, male; F, female; W, white Caucasian; B, Black; lcSSc, limited cutaneous systemic sclerosis

* With exception of ID 04033, all patients also fulfilled 1980 ARA classification criteria
^ Imaging or FVC <70% predicted (only 1 patient had FVC <70% predicted without imaging evidence of ILD
$ Arthropathy defined as synovitis or joint pain

Sample code	Antibody specificity	ANA stain IIF	Cytoplasmic stain on IIF	2013 ACR/EULAR*	Gender	Ethnic origin	Subtype	Overlap syndrome	Arthropathy $	Myopathy	ILD ^	SRC	MAL	RP	GORD	CAL	TEL	DU/DP	PAH
ANTI-eIF2B ANTIBODY POSITIVE																			
00339	eIF2B	Neg	FCS	Yes	M	W	dcSSc	No	N/A	No	Yes	No	No	Yes	No	No	No	No	Yes
00344	eIF2B	Neg	FCS	Yes	F	W	dcSSc	No	Yes	N/A	Yes	No	N/A	Yes	Yes	Yes	Yes	N/A	No
06449	eIF2B	Neg	FCS	Yes	F	W	dcSSc	No	Yes	N/A	Yes	No	Yes	Yes	Yes	No	No	No	No
06504	eIF2B	FS NS	FCS	Yes	M	W	dcSSc	No	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	No
06518	eIF2B	Neg	FCS	Yes	F	W	dcSSc	No	No	No	Yes	No	No	Yes	Yes	No	No	No	No
06779	eIF2B	FS, NS	FCS	Yes	F	B	dcSSc	RA	Yes	No	Yes	No	No	Yes	Yes	No	No	No	No
06790	eIF2B	Neg	FCS	Yes	F	W	lcSSc	RA	Yes	No	Yes	No	No	Yes	Yes	No	No	No	No
06922	eIF2B	Neg	FCS	Yes	F	W	dcSSc	No	N/A	Yes	N/A	Yes	Yes	Yes	Yes	N/A	Yes	N/A	No
07820	eIF2B	Neg	FCS	Yes	M	W	dcSSc	No	Yes	No	Yes	No	No	Yes	Yes	No	N/A	Yes	No
ANTI-SYNTHETASE ANTIBODY POSITIVE																			
08035	OJ	Neg	FCS	Yes	F	W	dcSSc	No	Yes	Yes	N/A	No	No	Yes	No	No	Yes	Yes	No
04219	PL12	Neg	FCS	Yes	F	W	lcSSc	No	No	No	Yes	No	N/A	Yes	Yes	No	No	No	No
06536	PL12	Neg	FCS	Yes	F	W	lcSSc	No	Yes	No	Yes	No	No	Yes	Yes	Yes	Yes	No	No
02954	PL12 and U1-RNP	Neg	FCS	Yes	F	W	dcSSc	No	N/A	N/A	N/A	No	N/A	N/A	N/A	N/A	N/A	N/A	Yes
00849	PL7	Neg	FCS	Yes	F	W	lcSSc	No	N/A	N/A	N/A	No	N/A	Yes	N/A	N/A	Yes	N/A	No
07836	PL7	Neg	FCS	Yes	F	W	lcSSc	No	Yes	No	Yes	No	No	Yes	Yes	No	No	Yes	No
05253	Zo	Neg	FCS	Yes	F	W	lcSSc	No	Yes	No	Yes	No	Yes	Yes	Yes	N/A	Yes	No	No
OTHER NOVEL SSc-SPECIFIC AUTOANTIBODIES																			
03540	RuvBL 1/2	FS NS	Neg	Yes	F	W	dcSSc	PM	N/A	N/A	N/A	No	N/A	Yes	Yes	N/A	Yes	No	No
04033	RuvBL 1/2	Neg	Neg	Yes*	F	W	lcSSc	SS	Yes	No	N/A	No	N/A	Yes	Yes	Yes	Yes	No	Yes

Figure 1. Detection of novel SSc-specific and anti-synthetase autoantibodies by immunoprecipitation.

Figure 1A. A schematic of the investigative findings following IPP within the “ANA negative” samples according to sample availability and IIF staining patterns.

Figure 1B. Detection of anti-EIF2B in SSc serum. The black arrow indicates 30kDA band present in anti-eIF2B samples. Lane 1: healthy control; Lane 2-10: anti-EIF2B positive samples (Lane 2, 00344; Lane 3, 00339; Lane 4, 06449; Lane 5, 06504; Lane 6, 06518, Lane 7, 06779; Lane 8, 06790; Lane 9, 06922; Lane 10, 07820).

Figure 1C. Detection of anti-RuvBL1/2 and anti-synthetase autoantibodies in SSc serum. The black arrows highlight bands indicative of antibody presence. Lane 1, healthy control; Lane 2, Anti-RuvBL1/2 (03540); Lane 3, Anti-RuvBL1/2 (04033); Lane 4, Anti-PL12 (02954); Lane 5, Anti-PL12 (04219); Lane 6, Anti-PL12 (06536); Lane 7, Anti-PL7 (00849); Lane 8, Anti-PL7 (07836); Lane 9, Anti-Zo (05253); Lane 10, Anti- OJ (08035).