Title: Anti-Eukaryotic Initiation Factor 2B Autoantibodies are Associated with Interstitial Lung Disease in Patients with Systemic Sclerosis

Zoe E. Betteridge, PhD, Felix Woodhead, MA MB BChir, PhD, Hui Lu, Gavin Shaddick PhD, Christopher C. Bunn PhD, Christopher P. Denton, PhD MD FRCP, David J. Abraham PhD, Roland M. du Bois MD, Mervyn Lewis, Athol U. Wells MD and Neil J. McHugh MBChB MD FRCP

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Corresponding Author:

Dr Zoe Betteridge

Pharmacy and Pharmacology, University of Bath, Bath, UK, BA2 7AY

Email: z.e.betteridge@bath.ac.uk

Tel: +44 1225 384271
Objective

Anti-nuclear autoantibodies are known to occur in 85-99% of Systemic Sclerosis (SSc) patients, with each SSc autoantibody correlating with a distinct clinical subset of patients. The objective of this study was to investigate novel SSc autoantibodies in the remaining autoantibody negative patients and establish clinical associations.

Methods

Serum samples and clinical data were collected from 548 SSc patients. Sera were tested for known SSc autoantibodies by routine serological techniques, with negative samples being further investigated by radiolabelled protein immunoprecipitation (IPP). Sera that immunoprecipitated a novel 30 kDa band were analysed by indirect immunofluorescence and IPP using depleted cell extracts to establish a common reactivity. Mass spectrometry (MS) was used to identify the novel autoantigen and findings were confirmed using commercial antibodies. Sera from 426 patients with other forms of connective tissue disease, 103 patients with rheumatoid arthritis, 114 patients with idiopathic ILD and 150 healthy controls were serotyped as controls.

Results

A novel autoantigen with a molecular weight of ~30 kDa was recognised by seven sera with SSc, six of whom had interstitial lung disease (ILD) and by no controls. Six of the patients had diffuse cutaneous involvement and four had overlap features with other autoimmune diseases. Immunodepletion experiments indicated that all samples targeted the same
autoantigen and MS identified the novel autoantigen as eIF2B (Eukaryotic Initiation Factor 2B).

**Conclusion**

We report a novel autoantibody (anti-eIF2B) in a small number of patients with SSc (approximately 1%) that is closely associated with diffuse cutaneous manifestations and the presence of ILD.
Systemic Sclerosis (SSc) is an uncommon disease characterised by microvascular damage and fibrosis of the skin and internal organs (1), with anti-nuclear autoantibodies (ANA) occurring in 85-99% of patients (2). The most common autoantibodies are anti-centromere (ACA) found in 7-40% of patients and anti-topoisomerase-1 (ATA, also known as anti-Scl-70) found in 9-42% of patients. Other SSc-specific autoantibodies include anti-RNA-polymerase-III, anti-U3-RNP/Fibrillarin, anti-Th/To and anti-U11/12. Additionally, SSc-associated autoantibodies such as; anti-Pm-Scl, anti-U1-RNP, anti-Ro/La and anti-Ku are found in SSc patients with overlapping autoimmune conditions including; Sjögren’s syndrome (SS), systemic lupus erythematosus (SLE) and myositis (2-4). Studies have demonstrated strong clinical associations between autoantibody class and disease phenotype (5) and whilst the SSc-specific autoantibodies are generally mutually exclusive, combinations of SSc-associated autoantibodies are more prevalent (2, 6). However, despite the identification of these autoantibodies, there still remains a group of SSc patients who appear to be autoantibody negative. Herein we report a novel autoantibody to eIF2B (Eukaryotic Initiation Factor 2B) in seven SSc patients who are negative for published SSc-specific autoantibodies.
Patients and Methods

Patient Cohort

Clinical data and sera were available from 548 SSc patients who fulfilled either the ARA and/or the LeRoy and Medsger criteria (7, 8). The study included 379 consecutive patients assessed for interstitial lung disease (ILD) at the Royal Brompton Hospital (RBH), mainly referred by the Royal Free Hospital. The remaining 169 patients attended the Royal National Hospital for Rheumatic Diseases (RNHRD). Clinical data were collected using standardised pro-formas and patients were classified as having either limited (lcSSc) or diffuse (dcSSc) cutaneous SSc according to LeRoy et al (8). The control population consisted of 211 patients with SLE, 42 patients with SS, 17 patients with SLE/SS overlap, 103 patients with rheumatoid arthritis (RA) (33 with ILD), 126 patients with myositis, 30 patients with undifferentiated connective tissue disease (UCTD) (defined by having symptoms suggestive of CTD but not fulfilling specific criteria), 114 patients with idiopathic ILD (defined by HRCT scan, or where not available, by the presence of bilateral basal inspiratory crackles on physical examination accompanied by reduced diffusing capacity on pulmonary function tests), and 150 healthy controls. Written consent to participate and provide biological samples was obtained from all subjects according to the Declaration of Helsinki, under the local ethical committee regulations.

Autoantibody measurement

Indirect immunofluorescence (IIF) was performed on all samples. For the RBH SSc patients, ACA were determined by IIF, ATA by counter-immune electrophoresis (CIEP) and anti-RNA-polymerase-III by INOVA ELISA. Samples negative for ACA, ATA and anti-RNA-polymerase-III
were further assessed by radiolabelled protein immunoprecipitation (IPP). All RNHRD SSc sera and controls were screened by both IIF and IPP.

**Indirect immunofluorescence**

IIF was performed on HEp-2 cells (Immunoconcepts, USA) using either fluorescein-labelled anti-Human IgG (Sigma, UK) (for patient samples) or fluorescein-labelled anti-rabbit IgG (Sigma, UK) (for the anti-eIF2Bβ control). Samples were screened at 1:40 and where anti-eIF2B positive, titred by serial dilution.

**Counter Immune Electrophoresis**

CIEP was performed as described previously (10) using lyophilised rabbit thymus extract (Pel-Freez, USA) and bovine spleen extract in PBS (Sigma).

**Protein Immunoprecipitation (IPP) using [³⁵S]-methionine**

10 µl sera was mixed with 2 mg protein-A-Sepharose beads (Sigma, UK) in IPP buffer (10 mM Tris-Cl pH 8.0, 500 mM NaCl, 0.1% v/v Igepal) for 30 min. Beads were washed in IPP buffer prior to the addition of 120 µl [³⁵S]-methionine labelled K562 cell extract (prepared from 15x10⁶ cells/ml). Samples were mixed at 4°C for 2 hr. Beads were washed in IPP buffer and TBS buffer (10 mM Tris-Cl pH 7.4, 150 mM NaCl) before being resuspended in sample buffer (SB) (Sigma, UK). After heating, proteins were fractionated by 9% SDS PAGE and analysed by autoradiography.

**Immunodepletion Experiments**
Duplicate samples containing 10 mg protein-A-Sepharose beads and 40 µl case-E or normal control serum (NS) were mixed for 30 min. Beads were washed in IPP buffer, and 150 µl [$^{35}$S]-methionine labelled K562 cell extract (prepared from 15x10⁶ cells/ml) was added to one aliquot. Aliquots were mixed at 4°C for 2 hr, before the supernatants were transferred to the corresponding duplicate and mixed at 4°C for 2 hr. The supernatant was stored at -80°C. IPP using 10 µl sera from anti-eIF2B positive patients and either 500 µl NS depleted or case-E depleted [$^{35}$S]-methionine labelled cell extracts were completed as described for IPP using [$^{35}$S]-methionine.

**Mass Spectrometry (MS)**

IPP was completed using 40 µl serum, 4 mg protein-A-Sepharose beads and 5 mM bis-(sulphosuccinimidyl)-suberate (Perbio, UK). Beads were incubated in 4 ml K562 cell extract (prepared from 28x10⁶ cells/ml) for a total of 4 hr at 4°C. Samples were resuspended in SB and heated. Proteins were fractionated by 10% SDS-PAGE and stained with Imperial Protein Stain (Perbio, UK). Bands were prepared for MALDI-TOF MS at the University of the West of England. Database matching using the ProteinLynx software required peptide coverage of over 20% with matching of the major theoretical and experimental peptide peaks.

**IPP-Blotting**

IPP was completed using either 40 µl sera (case-E, case-F, NS or serum containing known autoantibodies) or 20 µl polyclonal rabbit anti-eIF2BB antibody, 4 mg protein-A-Sepharose beads and 5 mM bis-(sulphosuccinimidyl)-suberate (Perbio, UK). Beads were incubated in 1 ml K562 cell extract (prepared from 28x10⁶ cells/ml) for a total of 2 hr at 4°C. Samples were resuspended in SB, heated and fractionated by 10% SDS-PAGE. Immunoprecipitates were
transferred to nitrocellulose and probed with rabbit polyclonal anti-eIF2BB antibody (1:500 dilution) for 90 min. Bands were detected using alkaline phosphatase conjugated goat anti-rabbit IgG (1:25000 dilution) (Sigma, UK) and BCIP/NBT liquid substrate (Sigma, UK).

**Statistical Analysis**

Clinical associations were derived from 2×2 contingency tables. Probabilities were calculated using Fisher's exact test except where the 2×2 contingency tables included a ‘0’, in which case p-values were calculated by 1-sided Barnards Test. Where applicable, data were expressed as odds ratios with 95% confidence intervals. Data were analysed using ‘R’ software.

**Results**

**Frequency of Known Autoantibody Specificities in SSc**

223 (58.8%) RBH SSc and 105 (62.1%) RNHRD SSc serum samples were positive for ACA, ATA or anti-RNA-polymerase-III. The respective frequencies are given in Table 1. The RBH cohort was enriched for patients suffering from ILD explaining the higher ratio of ATA to ACA compared to the RNHRD patients (RBH 2.7:1 vs RNHRD 0.4:1). Similarly, the association of ATA with dcSSc enriched the RBH cohort for dcSSc patients compared with the RNHRD patients (33.0% vs 17.2%).

IPP on the RNHRD samples, and the RBH the samples negative for ACA, ATA and anti-RNA-polymerase-III revealed a variety of autoantibodies recognised to occur in SSc or SSc-overlap including; anti-PM-Scl, anti-U1-RNP, anti-U3-RNP and anti-Ku. In addition, anti-tRNA synthetase antibodies were seen in six samples; four with anti-Jo-1 (anti-histidyl-tRNA
synthetase) and two with anti-PL-12 (anti-alanyl-tRNA synthetase). Additional analysis of the RNHRD cohort confirmed the presence of anti-Th/To in seven patients (4.1%), one of which had co-existing anti-Ro60. Three of these anti-Th/To positive patients (42.9%) had ILD, which whilst not statistically significant, demonstrated a similar trend to described previously (11).

**Identification of a novel SSc autoantibody**

Seven SSc sera (four RBH [A-D] and three RNHRD [E-G] patients) had a distinct IPP pattern (approximately 30, 40 and 53 kDa bands), that did not correspond to any previously identified SSc autoantigen. With the exception of one patient (G), who had co-existing anti-Ro60, this pattern was found to be mutually exclusive from other autoantibodies tested for by IIF or IPP. This pattern was not seen in immunoprecipitations using sera from disease or healthy control groups (p<0.001). Overall the prevalence of this autoantibody in the entire cohort was 1.3% (assuming the RBH ACA, ATA and anti-RNA-polymerase-I/III positive samples were negative for eIF2B) or 1.8% in the RNHRD cohort, all of which were screened by IPP.

**Autoantigen identification**

Immunodepletion studies demonstrated that the IPP pattern seen with the seven cases was due to the precipitation of the same autoantigen. When cell extracts were pre-depleted with NS, novel antigens were still visible after IPP with each of the patient’s samples (Figure 1A). However, after pre-depletion with case-E serum, and removal of the corresponding targets, the 30 and 40 kDa bands were absent, or greatly diminished, after subsequent IPP
by case A-G serum (Figure 1A). These results strongly imply that all seven sera target the same protein complex.

**Identification of eIF2B as the Target Autoantigen**

IPP and SDS-PAGE demonstrated the presence of approximately 30 and 60 kDa bands using serum from case-E (data not shown). MALDI-TOF MS and Swiss Prot analysis of the peptide fingerprints corresponded to Eukaryotic Initiation Factor 2B, subunit β (eIF2BB) and subunit δ (eIF2BD) respectively. These results were consistent when repeated on two separate occasions.

**Confirmation of eIF2B as the Target Autoantigen**

IIF of HEp-2 cells using the positive cases resulted in a fine cytoplasmic speckled pattern for all cases, with titres between 1:160 to 1:2560 (Table 2). This same pattern was seen on IIF using commercial anti-eIF2Bβ serum (Supplementary Figure 1). Additional confirmation of the target autoantigen was established by IPP-Blot. IPP was completed using a commercial antibody to eIF2Bβ, case-E and case-F sera, NS and sera containing known autoantibodies. When the immunoprecipitates were transferred to nitrocellulose and probed with the anti-eIF2Bβ antibody, bands were seen at the appropriate molecular weight with the eIF2Bβ, case-E and case-F immunoprecipitates. No bands were apparent with the NS or immunoprecipitates from patients with other known autoantibodies (Figure 1b). These results show that the novel precipitation pattern seen with SSc patients is due to autoantibodies directed against eIF2B.
Clinical Features of Patients with anti-eIF2B Autoantibodies

Clinical features of the seven patients with anti-eIF2B are given in Table 2. The presence of anti-eIF2B autoantibodies was associated with dcSSc compared to the anti-eIF2B negative SSc patients (85.7% (6 out of 7 patients) vs 31.1% (148 out of 476 patients), p<0.017 OR: 13.30 [1.59-111.44]). Two patients had evidence of co-existing myositis and two of RA/SSc overlap. Six patients had documented ILD by HRCT that appeared typical of scleroderma lung. The seventh patient did not have an HRCT, but did have a reduced pulmonary transfer factor (TLco 74% predicted) and was believed to have some pulmonary involvement, however due to the uncertain ILD diagnosis this patient was excluded from statistical analysis. The association of anti-eIF2B and ILD was borderline significant across the entire cohort (100% (6 patients) vs 63.4% (343 from 541 patients), p=0.05), despite the increased ILD bias of the cohort due to the inclusion of the RBH patients. When analysing the association of anti-eIF2B and ILD against the more representative RNHRD cohort, ILD was found to be a strongly associated risk factor in the anti-eIF2B positive patients (100% (6 patients) vs 25.9% (43 patients from 166) p<0.001).

Discussion

Here we report the identification of a novel autoantibody, anti-eIF2B, in seven SSc patients. Four patients formed part of a cohort of SSc patients assessed for ILD at a national unit, with the remaining patients attending a regional rheumatology centre. Anti-eIF2B autoantibodies are uncommon within the SSc cohort only being found in 1.3% of patients. However, whilst all of the RNHRD patients were screened for anti-eIF2B by IPP, the 223 RBH samples found positive for ATA, ACA or anti-RNA-polymerase-III were not screened by this method and not tested for anti-eIF2B. Whilst none of the RNHRD patients with anti-eIF2B had co-existing
ATA, ACA or anti-RNA-polymerase-III autoantibodies, it is possible that some additional patients in the RBH cohort are positive for anti-eIF2B, making the frequency higher than described.

The presence of anti-eIF2B corresponded with an increased risk of ILD, with six patients having definite ILD and the seventh having a reduced pulmonary transfer factor. Furthermore, six of the patients with anti-eIF2B had dcSSc. Over half of the anti-eIF2B positive patients had overlap features of either myositis or RA, although the autoantibody was not detected in any of the control cohorts, strongly inferring that the autoantibody is specific for SSc or SSc-overlap patients.

Previous reports have shown that the prevalence of autoantibodies to nuclear autoantigens in SSc patients ranges from 85-99% (2), with similar frequencies found in both the RBH and RNHRD cohorts. Interestingly, none of the seven anti–eIF2B positive sera had a positive ANA, but all had a cytoplasmic speckle on IIF; consistent with the cytoplasmic location of eIF2B. It may therefore be useful to investigate ANA negative SSc patients for the presence of anti-eIF2B autoantibodies in the appropriate clinical context.

Similarly to other cytoplasmic autoantigens, eIF2B is involved in protein translation. eIF2B is a multimeric protein consisting of 5 subunits that regulate a key step in protein synthesis (12). There have been reports of autoantibodies to other proteins in the eukaryotic protein initiation pathway, including anti-eIF3 in polymyositis (13) and anti-Agonaute/Su (eIF2C) in SLE (14). Interestingly, it has been demonstrated that Epstein-Barr virus expresses potentially immunogenic viral matches to all of the eIF2B subunits (15); and studies have
demonstrated 58% of SSc patients to have abnormal antibodies to EBV (16). It is therefore possible that anti-eIF2B autoantibodies are formed in selected SSc patients due to EBV molecular mimicry.

We report a new autoantibody, anti-eIF2B in 1-2% of SSc patients. This autoantibody is significantly associated with dcSSc and ILD, with a number of anti-eIF2B positive patients also having overlap features of myositis or RA. Sera from patients with anti-eIF2B autoantibodies are ANA negative, but have a cytoplasmic staining pattern by IIF, demonstrating anti-eIF2B to be the first SSc-specific cytoplasmic autoantibody. Further work is now required to understand why eIF2B is targeted by the immune system of these patients.

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