Anti-fibrillarin antibodies in systemic sclerosis

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Abstract

Objective. To investigate the nature and extent of organ involvement in anti-fibrillarin antibody (AFA)-positive patients within a UK systemic sclerosis (SSc) population.

Methods. We investigated 1026 consecutive patients with SSc. AFA was identified by the characteristic clumpy nucleolar and coilin body pattern of staining in interphase cells and staining of fibrillarin in metaphase cells by indirect immunofluorescence using HEp-2 cells. Identity of the 34-kDa fibrillarin protein was confirmed by immunoprecipitation from [³⁵S]methionine-labelled HeLa cell extract.

Results. AFA was detected in 42 patients (4.1%) with early disease onset (mean age 36 yr). Sixteen (38%) patients had limited cutaneous (lcSSc) and 26 (62%) diffuse cutaneous SSc (dcSSc). All eight Afro-Caribbean patients with AFA had dcSSc whereas the Caucasians were equally divided between dcSSc and lcSSc. Within the dcSSc subgroup, 54% had myositis, 35% had pulmonary hypertension, 15% had cardiac involvement and 23% had renal involvement.

Conclusions. AFA identifies young SSc patients with frequent internal organ involvement, especially pulmonary hypertension, myositis and renal disease. In contrast to previous reports, AFA was not restricted to dcSSc patients in Caucasians.

Key words: Systemic sclerosis, Anti-fibrillarin antibody, Myositis, Pulmonary hypertension, Renal.

Systemic sclerosis (SSc) is a heterogeneous autoimmune rheumatic disease characterized by Raynaud’s phenomenon and fibrosis of the skin and internal organs. The extent of skin or visceral fibrosis varies between affected individuals, as does the frequency of complications such as renal insufficiency, myositis and pulmonary hypertension [1, 2]. Although the previous classification of SSc into two major subsets, limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc), was based upon the extent of skin sclerosis [3], it is now appreciated that serological characteristics can provide valuable additional information by identifying patients at increased risk of specific organ involvement [4, 5]. The major autoantibodies in SSc sera occur in non-overlapping, mutually exclusive subgroups. They identify patients with distinct clinical subsets of scleroderma in terms of skin involvement, degree and severity of internal organ involvement and ultimately prognosis [6, 7].

Approximately 96% of SSc patients are positive for anti-nuclear antibody (ANA) when HEp-2 cells are used as substrate for ANA detection [5]. These ANA mostly recognize a range of well-characterized antigens, some of which are specific for SSc [8]. Although the pattern of nuclear and cytoplasmic fluorescence on HEp-2 cells is helpful in discriminating these antigens, the autoantibodies may be further characterized by analysis using cell extracts and purified antigens by counterimmunoelectrophoresis, enzyme-linked immunosorbent assay or radioimmunoprecipitation methods. Anti-centromere (ACA), anti-DNA topoisomerase-1 (ATA) and anti-RNA polymerases (ARA) are the commonest autoantibodies specific for SSc [4, 5, 9]. Anti-fibrillarin antibody (AFA) or anti-U3-RNP and anti-Th RNP are less common but also regarded as specific [10–14]. In polymyositis/SSc overlap, anti-PM-Scl is common but anti-Jo-1 and other aminoacyl tRNA synthetases occur infrequently [9, 15]. Antibodies to Ro, La or anti-U1-RNP may also be present as the only serological marker in a proportion of SSc patients but have little specificity for scleroderma.

Anti-fibrillarin (or anti-U3-RNP) antibody was first described by Bernstein et al. in 1982, occurring as the clumpy nucleolar pattern [16]. This antibody stains both the nucleoli and the coilin bodies, which is consistent with the known cellular distribution of the U3-RNP particle. The autoantibody also results in fibrillar (lace-like) staining of condensed chromatin in mitotic cells. It recognizes a highly conserved 34-kDa basic protein of small nucleolar ribonucleoprotein (RNP) particles called fibrillarin [17]. However, as this antibody occurs less frequently than the major SSc-specific autoantibodies, previous reports involved small numbers of patients. Previous studies on North American patient groups


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have suggested that AFA may be associated with pulmonary arterial hypertension and skeletal muscle disease, especially in young African-American males [10–13]. In the largest series reported to date, we demonstrate here the nature and extent of organ involvement in 42 AFA-positive patients who are likely to be representative of the UK SSc population. We confirm and extend the observations made in the previous North American studies.

Patients and methods

Patients

We studied 1026 consecutive patients with SSc who were evaluated at the Royal Free Hospital connective tissue disease clinic. All patients fulfilled the American College of Rheumatology classification criteria for definite SSc [4]. The patients were classified as having limited or diffuse cutaneous involvement according to LeRoy et al. [2], with diffuse skin involvement defined by skin tethering proximal to the elbows and/or knees, excluding the face. Clinical features were ascertained by review of medical records. During the same period, approximately 10 000 other patients were screened for ANA in the routine diagnostic immunology laboratory.

All scleroderma patients had electrocardiography (ECG), echocardiography, pulmonary function tests, high-kilovolt chest radiography and measurement of serum biochemical profile, creatinine clearance and 24-h urinary protein. Invasive investigations were performed when the clinical evaluation and baseline investigations suggested involvement of specific organs. The following criteria for organ system involvement were used. Pulmonary fibrosis was defined by the presence of typical reticular or amorphous interstitial abnormalities on high-resolution computed tomography of the thorax. Pulmonary hypertension was defined by ECG, echocardiography and/or right-side cardiac pressure determined by catheterization [18]. Pulmonary hypertension was defined by a resting pulmonary artery pressure (PAP) >25 mmHg or an exercise PAP >30 mmHg on Doppler echocardiographic examination or right-heart catheterization. Isolated pulmonary hypertension was defined as pulmonary hypertension in the absence of significant interstitial lung disease. Renal involvement was recorded if there was any history of hypertensive renal crisis attributable to SSc even if subsequent recovery was complete, or where there was persistent inexplicable renal impairment or proteinuria (more than 0.5 g in 24 h). Muscle disease was defined by at least two of the following: significant proximal weakness (power below MRC grade three); increased creatinine kinase; myopathic changes on electromyography; abnormal muscle biopsy.

ANA testing by indirect immunofluorescence

Sera were diluted 1:100 in phosphate-buffered saline (PBS) [150 mm NaCl, 10 mm phosphate (pH 7.2)] and screened by indirect immunofluorescence [19] using a HeLa-2 cell substrate (Bion, Park Ridge, IL, USA) with rabbit anti-human polyclonal fluorescein isothiocyanate conjugate (F0200; Dako, Ely, UK) and viewed at 400× magnification under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Anti-ENA estimation

Counterimmunoelectrophoresis for ENA was performed as previously described, using soluble extracts from human spleen and rabbit thymus acetone powder (Pelfreez Biologicals, Rogers, AR, USA) as antigen [20] and antisera of confirmed specificity [21].

Immunoprecipitation

Immunoprecipitation from [35S]methionine-labelled HeLa cell extract was performed essentially as described previously [22]. Briefly, 25 × 10 cm culture plates of subconfluent HeLa cells were cultured in methionine-free Minimum Essential Medium with trans-35S label (0.5 MBq/ml) (ICN Radiochemicals, Thame, UK) overnight. The cells were harvested by scraping and washing in ice-cold PBS. Following resuspension in 5 ml of immunoprecipitation buffer (IPP buffer) [500 mm NaCl, 10 mm Tris (pH 8.0), 0.1% Nonidet P40], cells were sonicated on ice (3 × 30 s) and centrifuged at 14 000 g for 15 min, and the supernatant was used as antigen.

For immunoprecipitation, 20 μl of patient serum was incubated with 0.5 ml of 4 mg/ml protein A–Sepharose CL4B (Sigma, Poole, UK) in IPP buffer overnight at 4°C. Antibody-bound Sepharose was washed three times with IPP buffer and resuspended in 200 μl of IPP buffer, combined with 50 μl of 35S-labelled HeLa extract for 2 h with occasional mixing. After four washes with IPP buffer, the immunoprecipitated proteins were denatured by boiling for 2 min in 40 μl sample buffer, fractionated on 8% polyacrylamide gels [23]. Gels were then fixed and enhanced by soaking for 1 h in Amplify (Nycomed-Amersham, Little Chalfont, UK), and precipitates were visualized by autoradiography.

Patient follow-up

The cohort represented 1026 consecutive cases of SSc attending the Royal Free Hospital Centre for Rheumatology. The median duration of disease for this cohort was 8 yr (range 0–54). For the patients identified as having AFA, the median duration was also 8 yr (range 0–25).

Statistical analysis

For comparisons between patient groups, frequencies of organ complications were subjected to the χ2 test and P < 0.05 was taken to indicate statistical significance.
Results

Forty-two patients with SSc (42/1026) and three additional non-SSc patients in whom AFA were detected in routine ANA screening had characteristic clumpy nucleolar and coilin body staining of interphase cells and staining of fibrillarin in metaphase cells (Fig. 1). All had high levels of AFA, with titres greater than 1/1000 on ANA testing. The 34-kDa fibrillarin protein was also detected in all 45 patients by immunoprecipitation from 35S-labelled HeLa cell extract (Fig. 2) but not in any of the additional SSc patients tested by this method. Of these 45 patients, 26 had dcSSc and 16 had lcSSc. Two had undifferentiated connective tissue disease (UCTD) and one had isolated Raynaud’s phenomenon, without clinical evidence of an associated autoimmune rheumatic disease (Table 1). AFA were present in 13 males and 29 females with SSc. Twenty-two per cent (8/36) of Afro-Caribbeans had AFA compared with 3.8% (3/90) of Caucasians in this SSc cohort (P < 0.00001); only one Asian and two Oriental patients were positive for AFA (Table 1). Within the Afro-Caribbean subgroup, all eight patients had dcSSc; none had lcSSc. The median age of onset of SSc was 36 yr; onset was preceded by symptoms of Raynaud’s phenomenon for a median duration of 3.3 yr.

Although there were more AFA-positive patients classified as dcSSc (62%) than lcSSc (38%), this division was accentuated when ethnic origin was taken into account. Within the Afro-Caribbean patients, all had dcSSc, and both Oriental patients and the Asian patient had dcSSc, whereas only 47% of Caucasians had dcSSc (P < 0.01). Thus, apart from one individual, the AFA-positive lcSSc subset was almost entirely Caucasian (Table 2).

Internal organ involvement was noted in 31 SSc patients: 14 had myositis, six had pulmonary fibrosis (two of whom also had pulmonary hypertension), 11 had isolated pulmonary hypertension, seven had cardiac disease and six had cardiac disease. Dividing the patients with internal organ involvement into lcSSc and dcSSc subgroups (Table 2) revealed that all the patients with myositis had dcSSc, five of the six patients with pulmonary fibrosis had dcSSc; nine of the 11 patients with isolated pulmonary hypertension had dcSSc, and six of the seven with renal disease and four of the six with cardiac disease had dcSSc. Thus, in the AFA-positive dcSSc subgroup, 35% had isolated pulmonary hypertension, 54% had myositis, 15% had cardiac involvement, 23% had renal involvement and 19% had pulmonary fibrosis (Table 2).

Of the Afro-Caribbean patients with AFA and dcSSc, three of eight (37%) had isolated pulmonary hypertension, whereas the figure for Caucasians was four of 15 (27%) (P not significant). Cardiac involvement affected three of eight Afro-Caribbeans with dcSSc compared with one of 15 Caucasians. Muscle involvement occurred in three of eight (37%) Afro-Caribbeans compared with eight of 15 (57%) Caucasians (P not significant). One Afro-Caribbean patient had renal disease compared with three of 15 (20%) Caucasians with dcSSc. Both Oriental patients had isolated pulmonary hypertension, myositis and renal involvement; the one Asian patient had dcSSc and pulmonary fibrosis.

The autoantibody profile of these patients was retested annually throughout the follow-up. The median disease duration for all SSc patients was 8 yr (range 0–54); for those with AFA it was also 8 yr (range 0–25).

![Fig. 1. Fluorescent antinuclear antibody patterns on HEp-2 cells, demonstrating the clumpy nucleolar and coilin body staining of interphase cells and staining of fibrillarin in metaphase cells. Magnification ×400.](image1)

![Fig. 2. Autoradiograph of 8% polyacrylamide gel showing the detection of autoantibody specificities by radioimmuno-precipitation with the 34-kDa fibrillarin in the first lane. Other SSc-associated autoantibodies are included for comparison.](image2)
All patients remained AFA-positive with a persistent clumpy nucleolar pattern by indirect immunofluorescence. Two patients died during follow-up; one had dcSSc and one had lcSSc. In both cases the cause of death was pulmonary hypertension.

**Discussion**

This study of the clinical relevance of AFA in 42 patients with SSc confirms and extends the findings of the two previous American studies, which reported 24 and 27 patients respectively [9, 10]. The overall frequency of AFA in SSc is approximately the same in all studies. Their presence is associated with a short mean duration of disease at diagnosis and they occur more frequently in Afro-Caribbeans than Caucasians. AFA have been reported as being associated with diffuse disease and internal organ involvement, a feature supported by this study. The striking features in our study were the young age of onset of SSc and the association of AFA with isolated pulmonary hypertension, myositis and renal disease. The frequency of other internal organ involvement, notably cardiac and pulmonary fibrosis, was less marked. As with other SSc-associated autoantibodies, there was an interesting association between ethnic group and the overall frequency of internal organ involvement [24–26]. However, within the dcSSc subset the frequencies of isolated pulmonary hypertension, pulmonary fibrosis, cardiac disease, myositis and renal disease were not significantly different between Afro-Caribbeans and Caucasians. Thus, as described previously, AFA identifies a subset of SSc patients within the Afro-Caribbean and Caucasian populations with a high frequency of specific internal organ involvement. Although both AFA-positive Oriental patients had myositis, renal involvement and isolated pulmonary hypertension, this number of patients is too few to allow us to draw firm conclusions.

The presence of isolated pulmonary hypertension is a poor prognostic feature and occurs more commonly in lcSSc, affecting approximately 15% of this subgroup [1]. The presence of AFA in lcSSc was not associated with increased risk of pulmonary hypertension. However, within the dcSSc subgroup in this study, AFA was a powerful independent risk factor, identifying a 35% probability of developing isolated pulmonary hypertension. Therefore, identification of this antibody at diagnosis merits investigation and monitoring of the patient for the development of cardiopulmonary complications. This has important clinical implications as isolated pulmonary hypertension is difficult to diagnose without screening, and so investigations which identify dcSSc patients who are at increased risk of this complication and who can be further investigated are particularly useful. The high frequency of renal disease in dcSSc patients with AFA is striking. Consequently, careful monitoring for the development of scleroderma renal crisis in such patients is essential.

The low mortality of patients with AFA was a surprising feature; only two out of the total of 42 patients died, or two out of 11 patients with isolated pulmonary hypertension, but none with renal disease. However with a longer duration of follow-up for AFA-positive patients, it is possible that mortality from established complications, such as pulmonary hypertension, will be greater. Thus, long-term follow-up is important to clarify the prognostic significance of AFA in SSc.

The mutually exclusive nature of the major SSc-specific autoantibodies—ACA, ATA and ARA—has been documented previously, with one report of coexisting ARA and ATA [27] and one of coexisting ACA and ATA [4]. In the present study, AFA were mutually exclusive of the above major SSc associated antibodies, a finding also reported by two other groups [10, 28]. However, in one large study of 27 AFA-positive patients, five (19%) had concomitant antibodies to ATA and one (4%) had concomitant antibodies to ATA, U1-RNP and Ro [11]. These discrepancies may reflect genetic or other differences between patients, or perhaps result from different assay techniques. Such differences emphasize the value of confirming serological studies in different patient populations.

**Table 1. Demography of SSc patients**

<table>
<thead>
<tr>
<th>Age at onset (yr)</th>
<th>Mean 36</th>
<th>Range 12–61</th>
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<tbody>
<tr>
<td>Sex</td>
<td>13 male</td>
<td>29 female</td>
</tr>
<tr>
<td>Ethnic origin</td>
<td>Caucasian 31</td>
<td>Afro-Caribbean 8</td>
</tr>
<tr>
<td>Diagnosis</td>
<td>dcSSc 26</td>
<td>lcSSc 16</td>
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<td>(including 3 with polymyositis/SSc overlap)</td>
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**Table 2. Subsets of SSc and internal organ involvement according to ethnic origin (n = 42)**

<table>
<thead>
<tr>
<th>Caucasian (n = 31)</th>
<th>Afro-Caribbean (n = 8) dcSSc</th>
<th>Oriental (n = 2) dcSSc</th>
<th>Asian (n = 1) dcSSc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>15 16</td>
<td>8 2</td>
<td>2 1</td>
</tr>
<tr>
<td>Isolated pulmonary hypertension</td>
<td>4 2</td>
<td>3 2</td>
<td>– 1</td>
</tr>
<tr>
<td>Pulmonary fibrosis</td>
<td>2 1</td>
<td>2 –</td>
<td>– –</td>
</tr>
<tr>
<td>Cardiac involvement</td>
<td>1 2</td>
<td>3 –</td>
<td>– –</td>
</tr>
<tr>
<td>Renal involvement</td>
<td>3 1</td>
<td>1 2</td>
<td>– –</td>
</tr>
<tr>
<td>Muscle involvement</td>
<td>9 –</td>
<td>3 2</td>
<td>– –</td>
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AFAs have been reported as being specific for SSC. These antibodies have not been identified in any other clinical condition in our diagnostic immunopathology laboratory. Although two of our patients had UCTD and one had Raynaud’s phenomenon, it is possible that they may progress to develop SSC. Likewise, AFA was identified in one patient with primary Raynaud’s phenomenon in a previous study [10]. Thus, regular monitoring for the development of SSC in AFA-positive patients is important.

The high proportion of Caucasians with lcSSc contrasts with that reported in previous studies. Whether this antibody defines a particular subset within lcSSc is unclear, but within this small number of 16 patients internal organ involvement does not appear to be different from that in the well-reported ACA-positive lcSSc subgroup [29]. Although the clinical profile of internal organ involvement was similar in the dcSSc subset to that in previous studies, it emphasizes the value of combining serological features with skin score estimation for disease subset classification. The present study suggests that neither skin score nor AFA positivity alone is sufficient to predict organ complications.

In summary, AFA (or anti-U3-RNP) is readily recognizable as a distinct ANA pattern that identifies young SSC patients with diffuse scleroderma at risk of developing internal organ involvement. As with other SSC-related autoantibodies, there is an association between ethnic origin, autoantibody frequency, disease subset and related organ complications [24–26].

References


