Antinuclear antibodies in systemic sclerosis

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Antinuclear autoantibodies are present in more than 90% of patients with systemic sclerosis (SSc). Some of these antibodies can be regarded as disease-specific: anticentromere antibodies, anti-topoisomerase I and anti-RNA polymerase I–III identify the three major serological subsets. Other important antibodies recognize nucleolar antigens, such as fibrillarin, Th/To and PM/Scl. All of these specific serological reactivities are mutually exclusive, have definite clinical associations and prognostic implications. This makes antinuclear antibodies a valuable tool in classifying and managing patients with SSc. SSc pathogenesis remains unclear and the role of autoantibodies is debated. Studies exploring their relationships with new emerging disease mechanisms are still in progress.

Systemic sclerosis (SSc) is a chronic autoimmune disorder characterized by microvascular injury and connective tissue fibrosis, involving the skin and a variable number of internal organs, mainly the lungs, heart, gastrointestinal tract and kidneys.

Antibodies against cellular self-components are a well-known hallmark of autoimmunity. It has been reported that more than 90% of SSc patients test positive for antinuclear antibodies (ANA) [1].

Some of these ANAs can be regarded as disease-specific, such as anticentromere antibodies (ACAs), anti-topoisomerase I (anti-topo I), anti-RNA polymerases (RNAP) and a group of antinucleolar antigens (**Tables 1 & 2**) [2]. These autoantibodies appear in a mutually exclusive fashion in most cases and each subset can be found in association with definite clinical features. Autoantibodies are helpful in characterizing different patient groups and giving important indications regarding diagnosis, disease course and prognosis [3].

In SSc patients, associations of these autoantibodies with human leucocyte antigen (HLA) class II alleles have been extensively studied. No definite HLA background has been correlated to SSc itself or any SSc-associated clinical manifestation, but a number of susceptibility haplotypes have been recognized as occurring in strong association with different autoimmune profiles [2].

The mechanism explaining the mutual exclusivity of some of these autoantibodies is unclear. They could be the expression of distinct pathological processes or otherwise a common environmental pathological injury could first break tolerance to different self-components, leading to altered antigen processing and presentation by immunocompetent cells in a mechanism also involving HLA restriction [4–6].

Whether these autoantibodies play an active role in disease pathogenesis or merely represent an epiphenomenon, their importance resides mostly in disease specificity and diagnostic and prognostic implications.

Disease-specific autoantibodies
Anticentromere antibodies

At least six centromeric nucleoproteins (CENP–A–F) are recognized by ACA-positive SSc sera. There are no reports of clinically relevant differences related to targeting different centromeric proteins and all ACA-positive sera are found to react with CENP-B protein. ACAs are detected easily by indirect immunofluorescence (IIF) on interphase and metaphase Hep-2 cells (immortalized cells originated from human laryngeal carcinoma): they give a speckled pattern referred as 'centromeric' and do not usually need any further confirmation by other laboratory methods. A solid-phase enzyme-linked immunosorbent assay (ELISA) with a CENP-B fusion protein exists and has been validated in sensitivity and specificity for use in daily clinical practice. ACA levels remain stable during the course of the disease and do not have clinical relevance [7].

ACAs are highly specific for SSc; they occur in approximately 15–30% of patients with frequencies varying according to ethnic differences, being higher in Caucasians than in Hispanics, Africans, African–Americans and Asians. Clinically, they are strongly associated with a limited cutaneous involvement, high incidence of peripheral vasculopathy, ischemic digital loss and calcinosis, Raynaud's phenomenon, esophageal dismotility, sclerodactyly and teleangectasia (CREST) syndrome, classified by LeRoy as a form of limited cutaneous SSc (lcSSc) [2]. In

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Table 1. Three major disease-specific serological groups.

Autoantibody	Recognized epitope	Systemic sclerosis subset	Clinical associations	Frequency	Ref.
Anticentromere	Centromeric nucleoproteins	Limited cutaneous	Calcinosis, Raynaud's phenomenon, esophageal dismotility, sclerodactyly teleangectasia syndrome, digital ulcers	15–30% More frequent in Caucasians	[7]
Anti-topoisomerase I	DNA topoisomerase I	Diffuse cutaneous	Pulmonary fibrosis	10–35% More frequent in Thai patients	[12,22]
Anti-RNA polymerase I–III	Isoform I and III of RNA polymerases	Diffuse cutaneous	Scleroderma renal crisis	5–25% Less frequent in the Mediterranean areas and Far-Eastern countries	[4,20,21]

patients with apparently isolated Raynaud's phenomenon, the finding of ACA reactivity in sera should prompt the clinician to suspect SSc [7].

ACA-positive patients show lower frequencies of interstitial pulmonary fibrosis compared with other groups of SSc patients; they also have better survival rates. This serological subset is indeed considered to be slowly progressive, with the cutaneous involvement being stable over time and digital tip ulcers being the most frequent severe complication. A major cause of death in patients with SSc, and in particular in those with limited cutaneous involvement, is pulmonary arterial hypertension (PAH) without radiological signs of fibrosis and restrictive lung disease on pulmonary function tests. There are many new treatments for PAH and it is extremely important to recognize this complication as early as possible. This can be obtained by strict monitoring of these patients by indices proven to be good predictive factors, such as CO-diffusing capacity (DLCO) and the forced vital capacity (FVC)/DLCO ratio in addition to systolic pulmonary arterial pressure on an echocardiogram [8].

It has been demonstrated that ACAs may also exert anti-endothelial cell activity, thus showing that surface expression of the target of a classical SSc-associated antibody may occur in cells with a crucial pathogenic role, such as endothelial cells [9]. Antiendothelial cell antibodies (AECAs) have been associated with the development of manifestations such as PAH and digital ischemia in SSc. *In vitro*, they are able to mediate many effects such as vascular injury through antibody-dependent cytotoxicity and induction on endothelial cells of adhesion molecules, such as E-selectin, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule

(VCAM)-1, thus representing a possible explanation for vascular hyper-reactivity and consequent mononuclear migration to the inflamed tissue [10].

Anti-topoisomerase I

A basic, heat-labile, chromatin-associated, non-histone 70 kD protein (Scl70) was described in 1979 as the target of a group of human ANAs present in SSc sera and was named anti-Scl70 [11]. Further studies clarified that these autoantibodies recognize several epitopes of different molecular weights on the central and C-terminal portions of the enzyme topoI (topo I). In IIF, anti-topo I antibodies can show a variable and nonspecific staining pattern, which is homogeneous, speckled and also nucleolar. They are preferentially detected in routine laboratory assays by immunodiffusion (ID) on calf or rabbit thymus extracts. Other techniques include immunoblotting (IB), immunoprecipitation (IP) and ELISA with recombinant proteins. The latter, despite being less specific than ID, is widely used owing to its cost effectiveness and because it offers the advantage of a semiquantitative analysis [12].

Anti-topo I are specific and helpful in discriminating SSc from other connective tissue diseases (CTDs) and primary Raynaud's phenomenon.

As with ACAs, when a patient evaluated for Raynaud's phenomenon tests positive for anti-topo I, this subject has a very high risk of developing SSc [7].

Frequency ranges from 10 to 35%, with racial and geographic variations, being extremely common in SSc patients from Thailand [8]. Anti-topo I-positive patients are at high risk of developing diffuse cutaneous SSc (dcSSc). A strong clinical association links

Table 2. Main autoantibodies targeting nucleolar antigens in systemic sclerosis.

Autoantibody	Recognized epitope	Clinical associations	Frequency	Ref.
Anti-B23	Nucleophosmin	Limited cutaneous involvement, pulmonary arterial hypertension. Often associated with AFA	Rare	[23]
Anti-NOR90	Human upstream binding factor (NOR)	Limited cutaneous involvement	Rare	[24]
AFA	Fibrillarin, component shared by all of the box C/D sno-RNPs	Diffuse cutaneous involvement, arthritis, pulmonary hypertension	4–22% More common in African-Americans	[25]
Anti-U3-snoRNP	U3-snoRNP, one of the box C/D components	Diffuse cutaneous involvement. Often associated with AFA	Unknown	[25]
Anti-Th/To	RNase MRP and RNase P	Limited cutaneous involvement, esophageal involvement, pulmonary fibrosis and scleroderma renal crisis	2–5% More common in Japanese patients	[26]
Anti-PM/Scl	11–16 peptides with a range of MW from 20 to 110 kDa and with PM-Scl-100 and -75 being the most antigenic	PM/Scl overlap syndrome, limited cutaneous involvement	24% in PM/Scl overlap, 3% in SSc	[27]

AFA: Aflatoxin; NOR: Nucleolar organizer regions; PM/Scl: Polymyositis/scleroderma; snoRNP: Small nucleolar ribonucleoprotein.

anti-topo I to the development of interstitial lung disease, with a higher rate of decline in pulmonary function tests compared with other serological subsets [12].

There are several recent studies reporting fluctuations of anti-topo I antibody levels in serial samples. By using a very specific assay for immunoglobulin (Ig)G isotypes of anti-topo I, it has been shown that increased serum levels correlate positively with pulmonary function and skin score, sometimes even predicting flares of skin disease [13]. Another study has shown that the disappearance of anti-topo I may predict a more favorable outcome, even though changes in antibody levels were independent of clinical manifestations [14]. It should be noted that these results were obtained by research assays only and so far there is no evidence that antibody titer fluctuations detected using commercially available kits have any clinical correlation.

The survival rate in this group of patients is worse than in the ACA-positive subset, and lung involvement with subsequent ventricular failure is one of the most important causes of death in SSc [8]. A Japanese study reports an increased renal vascular resistance in anti-topo I-positive patients. This is a feature that may correspond to renal changes found in the kidneys of patients affected by scleroderma renal crisis (SRC), another SSc life-threatening complication [15]. However, studies confirming the association between anti-topo I and SRC are lacking.

It has been demonstrated that anti-topo I are strongly correlated with the presence of antifibroblast antibodies and that anti-topo I themselves exert an antifibroblast activity by reacting with a still unidentified membrane epitope [16]. Further studies should clarify whether this phenomenon is mediated by a mechanism of cross-reactivity or if it is the topo I itself brought to the fibroblast surface after some perturbation. Antibodies to membrane structures on fibroblasts are able to induce *in vitro* activation, proinflammatory mediator production and ICAM-1 upregulation by fibroblasts, thus claiming a role in perpetuating inflammation and inducing fibrosis [17]. Recently, a 100 kDa protein expressed on the endothelial cell surface and a target for IgG antibodies from anti-topo I-positive patients was identified as DNA topo I itself, suggesting that membrane expression of this protein can occur and have a functional role [18].

Anti-RNA polymerase I–III
Antibodies to RNAP I and III can be found in patients with SSc and are highly disease specific. The isoform I has nucleolar localization, while the isoform III resides in the nucleus. In IIF, these autoantibodies show a variable nucleolar and speckled pattern and they should not be classified strictly among the antinucleolar antibodies [2]. The gold standard technique to ascertain the presence of anti-RNAP in patient sera is IP, which is not so suitable for routine

detection in clinical practice as it is time consuming and requires radiolabelled cell extracts. An immunodominant epitope on RNAP III has been identified, obtained in a recombinant form and employed for testing SSc sera in an ELISA whose analytical accuracy and clinical specificity has been validated recently [19]. Anti-RNAP I and III reactivities are usually associated with each other and are the most disease-specific markers, preferentially detected in patients with a diffuse cutaneous involvement. Some patients, along with anti-RNAP I–III, can recognize subunits of the II isoform, and even this serological profile is highly specific for dcSSc. Anti-RNAP I–III are mutually exclusive with respect to ACA and anti-topo I [4]. Reactivity to anti-RNAP I–III or I–II–III in patients' sera has been associated clinically with the male sex, an older age of disease onset and a lesser extent of peripheral vascular and pulmonary involvement, but a higher frequency of cardiac and renal involvement compared with ACA- or anti-topo I-positive patients [20]. In particular, at least in Caucasian patients from the USA and UK, the incidence of SRC is significantly higher in anti-RNAP I–III dcSSc patients compared with anti-topo I-positive dcSSc patients [4]. By a preliminary ELISA evaluation, it seems that an increase in antibody levels may precede the development of SRC. The autoantibody titer has also been correlated with the course of skin involvement [19].

In Italy, the frequency of anti-RNAP I–III is consistently lower than in Caucasian SSc patients from the USA and UK and is closer to that reported in Far-East populations [21]. This low occurrence correlates with the low prevalence of SRC in Italian [22] and Japanese [15] SSc patients. The possibility of testing this autoantibody with ELISA will allow the study of large series and will clarify clinical associations, even in populations with low prevalence of anti-RNAP I–III.

Antinucleolar antibodies
Nucleolar proteins are specific targets for autoantibodies from SSc sera (Table 2). Some are quite rare, such as anti-B23 [23] or anti-Nucleolus organizer region (NOR)90 [24], while others, such as antifibrillarin (ATA) [25], anti-Th/To [26] and anti-polymyositis/scleroderma (PM/Scl) [27], are relatively common and have important clinical associations in definite SSc and SSc overlap syndromes.

Small nucleolar (sno) ribonucleoproteins (RNPs) are macromolecular complexes of RNA and proteins located in the nucleolus and involved in ribosome biogenesis. SnoRNPs are dynamic structures that many stressor agents (irradiation, chemotherapeutic toxins, mercury exposure, heat shock or activation of death receptors) may involve in the setting of an autoimmune process leading, ultimately, to autoantibody production [28]. Anti-snoRNPs give a clumpy or bright nucleolar IIF pattern and are identified mainly by IP or IB techniques.

Antifibrillarin & anti-U3-snoRNP
Fibrillarin is a component of box C/D snoRNPs shared by all of this class components (U3-, U8- and U22-snoRNPs). AFA can occur in various CTDs, but are most frequently found in SSc. AFA-positive patients represent 9–52% of the ANA-positive group of SSc patients [25]. They are more frequent in African–American patients and define a severe course of disease with diffuse skin sclerosis, esophageal involvement and a high risk of developing arthritis and PAH [8]. Although rare, single reactivity against the box C/D component U3-snoRNP alone can also be detected in SSc sera [25].

Anti-Th/To
These autoantibodies are found in 2–5% of patients, with the highest frequency in Japanese series. They recognize two RNA-processing enzymes, RNase MRP and RNase P, with ten associated proteins, and are more common among patients with a limited cutaneous involvement. The anti-Th/To-positive subset shows more subtle skin changes than ACA-positive lcSSc patients, with puffy fingers being a common finding. Peripheral vascular and gastrointestinal disease are less prominent, but there is a higher risk of developing complications more frequently found in dcSSc patients, namely pulmonary interstitial fibrosis and SRC. The occurrence of these clinical findings worsens the prognosis of anti-Th/To-positive patients compared with the ACA-positive group [26].

Anti-PM/Scl
PM/Scl identifies a RNA-processing group of 11–16 peptides, the 'human exosome', with molecular weights ranging from 20 to 110 kDa, by which they have been identified in IP and IB assays. PM/Scl-100 and -75 have shown the greatest antigenicity because they are recognized by the most positive sera and are therefore employed as

antigens in the more commonly used ELISA tests. These autoantibodies are important markers of the PM/dermatomyositis (PM/DM) SSc overlap syndrome, where they can be found in approximately 24% of cases. They can also be detected less frequently in SSc patients and PM/DM patients without overlapping features, with frequencies of 3 and 8%, respectively. Clinically, anti-PM/Scl patients with an overlap syndrome show a milder course of both diseases and a good response to steroid treatment [2].

Other antinuclear antibodies
Anti-RNAP II
Antibodies to the isoform II of RNAP target the three major subunits of this enzyme: IIo, which is highly phosphorylated; IIa, an unphosphorylated form; IIc, which results from cleavage of the former subunits. In SSc, anti-IIo, anti-IIa and anti-IIc can all be recognized together with the isoforms I and III. Anti-RNAP II reactivity may be present alone or in association with anti-topo I.

In the latter cases, the subunit recognized is more frequently represented by IIo. Anti-RNAP II antibodies in SSc do not show distinct clinical associations and can also be detected in mixed CTD (MCTD) and systemic lupus erythematosus (SLE) [4].

Anti-Ro
Anti-Ro autoantibodies are more commonly detected in Sjögren's Syndrome (SSj) or SLE, but they can also be found in SSc, in particular if the autoantigen recognized is the Ro52 subunit. Its presence can be indicative of a higher incidence of sicca syndrome symptoms [2].

Anti-U1-RNP
Anti-U1-RNP is a well known marker of MCTD, but it can also be found in definite SSc, especially if directed against the 70 kD subunit of the molecule. Clinically, anti-U1-RNP-positive SSc patients have limited cutaneous disease and joint involvement [29].

Executive summary

Antibodies in systemic sclerosis
- Antibodies to nuclear self-components are present in more than 90% of systemic sclerosis (SSc) patients. Some autoantibodies are specific and can be regarded as markers of the disease. These include anticentromere antibodies (ACA), anti-topoisomerase I (anti-topo I), anti-RNA polymerase (anti-RNAP)I–III and some of the so-called antinucleolar antibodies (ANoA).
- The importance of disease-specific autoantibodies resides in the fact that they are mutually exclusive in a single patient and possess definite clinical and prognostic associations. Therefore, they represent a precious tool for clinicians.

Disease-specific markers
- ACAs are associated with a limited cutaneous involvement, calcinosis, Raynaud's phenomenon, esophageal dismotility, sclerodactyly and teleangectasia (CREST) syndrome and peripheral vasculopathy. As for other patients with limited cutaneous SSc, ACA-positive patients may be at risk of developing isolated pulmonary arterial hypertension. Nevertheless, they have a good prognosis.
- Anti-topo I are more common in the diffuse cutaneous subset, and are associated with the development of pulmonary fibrosis. They have a worse prognosis compared with ACA-positive patients.
- Anti-RNAP I–III antibodies are the most specific marker of SSc and are associated with diffuse cutaneous involvement. They are also associated with scleroderma renal crisis (SRC). Survival rates have now been improved by treatment of SRC with angiotensin converting enzyme-inhibitors.
- Among the targets of ANoAs there are fibrillarin, U3-small nucleolar ribonucleoprotein and RNAse MRP/RNAse P (Th/To). Which are mutually exclusive with each other and the other three major disease-specific serological groups. Clinical associations are diffuse cutaneous involvement and pulmonary hypertension for antifibrillarin and limited cutaneous involvement, pulmonary fibrosis and SRC in anti-Th/To-positive patients.
- Other ANoAs are the rare anti-B23 and anti-nucleolus organizer region (NOR)90, and anti-polymyositis/scleroderma (PM/Scl), associated with PM/Scl overlap syndrome.
- Many other antinuclear autoantibodies can be found, although they are less specific for SSc.

Autoantibodies with potential direct pathogenic roles
- Several autoantibodies might be candidates for a direct pathogenic role. Among them, ACAs and anti-topo I have been reported to react with antigens on the surface of endothelial cells and fibroblasts.

Future perspectives in SSc
- B cells are now claimed to play an important role in SSc. Autoantibodies, even if not pathogenetic themselves, are a direct expression of B-cell activation and may represent a good instrument to shed light on the mechanisms of disease and to monitor the effects of novel therapeutic agents.

Anti-Ku

Ku is a chromatin-associated heterodimer that is composed of two subunits, 70 and 80 kD in weight, respectively. Anti-Ku autoantibodies are rare and reports on their clinical significance vary depending on the ethnicity of the population considered. They have been described in SSc, frequently in overlap with PM/DM or SLE, and associated with Raynaud's phenomenon, myositis, arthritis and pulmonary hypertension [30].

Antiheterogenous nuclear ribonucleoprotein I

Among the abundant group of heterogeneous nuclear (hn) RNPs, various components have been demonstrated to elicit autoimmune responses in a wide spectrum of rheumatic diseases; autoantibodies to hnRNP I, also named polypirimidine tract-binding protein, are associated specifically with SSc. Anti-hnRNP I can be found in all subsets, but is more frequently found in patients with lcSSc. Interestingly, hnRNP I differs from the other hnRNPs in its perinucleolar localization [31].

Conclusions

Genetic, environmental and stochastic factors may influence which antigen activates an autoimmune response within a single patient with SSc. Although the mechanistic implications of such observations remain uncertain, the diagnostic and prognostic meaning of ANAs is undisputed.

Sensitive and specific tests are now available in daily clinical practice, making detection and titer measurements easily accessible and providing a useful clinical tool for patient management.

Future perspective

Autoimmunity, flogosis and fibrosis are expressions of SSc physiopathology. Processes that could explain them in a single theory can provide pathogenetic information, useful in the identification of therapeutic targets. This issue is crucial in SSc, whose therapeutic armamentarium is still limited. Altered B-cell functions occur via various pathways, both in SSc patients and in the tight-skin mouse, an experimental model of SSc, leading to breakdown of tolerance with consequent autoantibody production. Furthermore, chronically activated B lymphocytes can produce interleukin-6 and transforming growth factor-β, which in turn induces increased collagen production by fibroblasts. Attention in the near future will be paid to the role of B-cell-targeted therapies in SSc. Disease markers such as autoantibodies may, albeit indirectly, give important clues when monitoring the effects of new therapeutic agents in this multifaceted disease [32].

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