Circulating Anti-Nuclear Antibodies in Systemic Sclerosis: Utility in Diagnosis and Disease Subsetting

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The presence of circulating anti-nuclear antibodies (ANAs) is a hallmark of immune dysregulation in patients with systemic sclerosis (SSc). Currently, a variety of SSc-specific ANAs, including anticentromere, anti-topoisomerase I, and anti-RNA polymerase III antibodies, have been well characterized, and their commercial kits are available worldwide. Since these autoantibodies are specifically detected in SSc patients and are associated with unique sets of disease manifestations, they are widely used in routine clinical practice for diagnosis, clinical subgrouping, and prediction of future organ involvements and prognosis. In addition, SSc-specific ANAs are also useful in predicting future development of SSc in patients with Raynaud’s phenomenon without any sclerodermat skin changes, because their production often precedes onset of SSc symptoms. Application of circulating SSc-specific ANA measurement to clinical practice has greatly improved patient care, but utility of the autoantibody testing could be maximized by combining other clinical information, such as degree and extent of skin thickness and disease duration. (J Nippon Med Sch 2017; 84: 56–63)

Key words: anti-nuclear antibody, biomarker, diagnosis, scleroderma, systemic sclerosis

Introduction
Systemic sclerosis (SSc) or scleroderma is a connective tissue disease characterized by excessive fibrosis, microangiopathy, and immune dysregulation, including autoimmunity and chronic low-grade inflammation. Multiple genes contribute to disease susceptibility, but environmental exposures are likely to play a major role in causing and progressing the disease. Since clinical presentation in patients with SSc is highly variable, disease subgrouping as well as prediction of future organ involvement and prognosis are extremely important in clinical setting. One of the distinctive hallmarks of the immune dysregulation is the presence of circulating autoantibodies reactive with various cellular components. The majority of disease-associated autoantibodies in SSc patients are anti-nuclear antibodies (ANAs) that target proteins that play essential roles in transcription, splicing, and cell division. It has been shown that ANA specificities specifically detected in SSc patients are associated with unique disease manifestations. Therefore, individual ANAs are attractive biomarkers in routine rheumatology practice. This review features the spectrum of SSc-specific ANAs and their clinical utility.

SSc-specific and SSc-related ANAs
ANAs detected by indirect immunofluorescence (IIF) technique are a hallmark of SSc, and are found in >95% of the patients. The majority of nuclear autoantigens recognized by SSc sera have been already identified to date. ANAs detected in SSc sera can be divided into two groups, including SSc-specific ANAs, which are specifically detected in SSc patients, and SSc-associated ANAs, which are found in patients with SSc but also in those with non-SSc connective tissue diseases. Currently, at least 10 ANA specificities specific to SSc have been reported and well characterized. These include anticentromere, anti-topoisomerase I (topo I), anti-RNA polymerase (RNP) III, anti-U3 ribonucleoprotein (RNP), anti-Th/To, anti-U11/U12 RNP, anti-PM-Scl, anti-Ku, anti-RuvBL1/2, anti-U1 RNP antibodies. These SSc-specific ANAs are detectable in >80% of SSc patients. Two classic autoantibodies discovered in the late 1970’s are anti-topo...
Anti-Nuclear Antibodies in Scleroderma

Table 1 Staining patterns on IIF and clinical correlation of SSc-specific ANAs

<table>
<thead>
<tr>
<th>ANA specificity</th>
<th>Staining pattern on IIF</th>
<th>Disease subset</th>
<th>Organ manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anticentromere (ACA)</td>
<td>Discrete speckled</td>
<td>lcSSc</td>
<td>PAH, DU in late disease</td>
</tr>
<tr>
<td>Anti-topo I</td>
<td>Speckled (with or without nucleolar)</td>
<td>dcSSc</td>
<td>ILD, DU in early disease</td>
</tr>
<tr>
<td>Anti-RNA polymerase III</td>
<td>Speckled (with or without nucleolar)</td>
<td>dcSSc</td>
<td>Rapid progression of skin thickening, SRC, GAVE, Malignancy at diagnosis</td>
</tr>
<tr>
<td>Anti-U3 RNP</td>
<td>Nucleolar</td>
<td>dcSSc/lcSSc</td>
<td>ILD, PAH, SRC, Lower GI involvement in early disease, Myopathy</td>
</tr>
<tr>
<td>Anti-Th/To</td>
<td>Nucleolar</td>
<td>lcSSc</td>
<td>ILD</td>
</tr>
<tr>
<td>Anti-U11/U12 RNP</td>
<td>Speckled</td>
<td>lcSSc (myositis overlap)</td>
<td>Myositis, DM rash</td>
</tr>
<tr>
<td>Anti-PM-Scl</td>
<td>Nucleolar</td>
<td>lcSSc (myositis overlap)</td>
<td>Myositis</td>
</tr>
<tr>
<td>Anti-Ku</td>
<td>Speckled</td>
<td>lcSSc (myositis overlap)</td>
<td>Myositis</td>
</tr>
<tr>
<td>Anti-RuvBL1/2</td>
<td>Speckled</td>
<td>dcSSc (myositis overlap)</td>
<td>Myositis</td>
</tr>
<tr>
<td>Anti-U1 RNP</td>
<td>Speckled</td>
<td>lcSSc (MCTD)</td>
<td>Inflammatory arthritis, Myositis, PAH</td>
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I antibody and anticentromere antibody (ACA). Another group of antibodies, including anti-U1 RNP, anti-Ku, anti-PM-Scl antibodies, were first identified using double immunodiffusion technique. The remaining ANA specificities were discovered using RNA or protein immunoprecipitation (IP) assay. SSc-specific ANAs target various nuclear components involved in essential cellular processes, such as cell division, splicing, and transcription. These autoantibodies are rarely seen in patients with other connective tissue diseases without SSc features and thus are important diagnostic markers. In addition, detection of SSc-specific ANAs is clinically useful in classifying SSc patients into subtypes that are almost exclusively associated with characteristic clinical phenotypes (Table 1). SSc-specific ANAs are usually present at diagnosis of SSc or even precede appearance of SSc-related clinical manifestations such as Raynaud’s phenomenon. In addition, these autoantibodies do not switch from one antibody to another, and typically remain detectable throughout the disease course, regardless of the treatment regimen. Patients rarely have two or more SSc-specific ANAs together, indicating mutual exclusiveness.

On the other hand, SSc-associated ANAs include antinucleolar organizing region 90 (NOR-90) and anti-B23 antibodies. Anti-NOR-90, also termed the human upstream binding factor of RNAP I, was first reported in patients with cancer, but later in patients with connective tissue diseases, including SSc, Sjögren’s syndrome, and rheumatoid arthritis. B23 is a nucleolar phosphoprotein overexpressed in many cancer cells, and is also targeted by cancer sera. Anti-B23 antibody is detected in <11% of SSc patients, and is associated with pulmonary arterial hypertension (PAH), often coexisting with anti-U3 RNP or anti-U1 RNP antibodies. Anti-SSA/Ro and anti-SSB/La can occur in SSc patients, but are usually associated with concomitant Sjögren’s syndrome. Recently, it has been shown that tripartite motif family of protein 21 (TRIM21) or Ro52, which is one of components of the SSA/Ro autoantigen, is targeted by sera from patients with various connective tissue diseases in the absence of anti-SSA/Ro antibody. A recent multicenter cohort study involving 963 patients with SSc has found that anti-TRIM21 antibodies are present in 20% of the patients and are associated with interstitial lung disease (ILD) and overlapping features with other connective tissue diseases.

There are a small proportion of SSc patients who are negative for ANA by IIF. In a recent cohort study involving 3,249 patients with SSc from a multicenter registry in North America, only 6.4% were negative for ANA. ANA-negative SSc patients constitute a distinct subset with a greater proportion of males, less frequent and less severe vasculopathy, and more frequent lower gastrointestinal (GI) involvement.
Clinical Correlations of Individual SSc-specific ANAs

1. Anticentromere Antibody (ACA)

Moroi and colleagues first described ACA, which was originally associated with the CREST (Calcinosis, Raynaud’s phenomenon, Esophageal hypomotility, Sclerodactyly, and Telangiectasia) variant of SSc. ACA recognizes the kinetochore lesion of individual metaphase chromosomes, and the autoimmune targets are now identified as centromere protein (CENP)-A, B, and C. The frequency of ACA in SSc patients has been reported to be 20–30% in many ethnic groups. ACA typically produces discrete speckled staining pattern on IIF, but use of chromosomal spreads as the substrate is necessary to confirm its presence. ACA is sometimes detected in patients with primary Sjögren’s syndrome or primary biliary cirrhosis as well as in individuals without an apparent connective tissue disease, who are almost always elderlies. The natural history of ACA-positive SSc patients includes long-standing Raynaud’s phenomenon followed by appearance of puffy fingers after a variable period of time, ranging from a couple of months to 10 or more years. Patients with ACA are almost always classified as having limited cutaneous SSc (lcSSc), a term which has now replaced CREST syndrome. Severe ILD, cardiomyopathy or sclerodema renal crisis (SRC) almost never occur in ACA-positive patients, but 10–20% develop PAH later in the course of the disease. In this regard, evidence-based DETECT algorithm for identification of SSc patients at high risk for developing PAH includes ACA as one of the useful characteristics.

2. Anti-topoisomerase I (Topo I) Antibody

Anti-topo I antibody was first discovered by double immunodiffusion assay as an antibody reactive with a 70-kDa protein (Scl-70), which was later identified as an enzyme that catalyzes relaxation of supercoiled double-stranded DNA, termed topo I. Anti-topo I antibody is detected in 20–30% of SSc patients in many ethnic groups, and is highly specific to SSc. The coexistence of anti-topo I and other SSc-related ANAs is rare (~1%). Almost two-thirds of anti-topo I-positive patients have diffuse cutaneous SSc (dcSSc), but progression of skin thickening is slower than those with anti-RNAP III antibody. ILD occurs in >70% of patients with anti-topo I antibody during the disease course, and ~25% develop severe disease which requires oxygen supplementation. The patients with severe ILD die of this complication at an average of 10 years after onset of SSc. Since ILD is now the major cause of death in SSc patients, anti-topo I antibody is considered to be a marker for poor prognosis. Nevertheless, progression and prognosis of ILD in patients with anti-topo I antibody are variable, although anti-topo I antibody titer is not useful in predicting a risk for developing severe ILD. Patients with anti-topo I antibody rarely develop PAH, but pulmonary hypertension secondary to ILD is fairly common later in the course of the disease. Other important organ involvements associated with anti-topo I antibody include cardiomyopathy and peripheral vascular complications, such as digital ulcer (DU) and gangrene, particularly early in the disease course.

3. Anti-RNA Polymerase III (RNAP III) Antibody

A series of autoantibodies to RNAPs were identified by protein-IP assay. SSc sera contain autoantibodies directed against the RNAP I, II, and III in different combinations, which fall into 4 groups: those reactive with RNAP I, II and III, those with RNAP I and III, those with RNAP III alone, and those with RNAP II alone. Antibodies to RNAP II alone, which specifically target an active form, is considered as an SSc-associated ANA, because they are detected in patients without SSc as well as in those with SSc in association with anti-topo I antibody. Instead, antibodies reactive with RNAP III, which are recognized commonly by the remaining groups, are most often regarded as the SSc-specific ANA. Sera positive for anti-RNAP III antibody produce a speckled staining pattern on IIF, but also produce nucleolar staining if anti-RNAP I antibodies coexist. The frequency of anti-RNAP III antibody in SSc patients varies among ethnic groups: higher frequency in North American Caucasian and UK patients (20–25%) in comparison with French or Japanese patients (5%). Age at onset is older and the proportion of males tends to be higher in patients with anti-RNAP III. Nearly all patients with this antibody have dcSSc with rapidly progressive skin thickening. Despite that, many patients experience rapid regression of skin thickening over time even without treatment.

Patients with anti-RNAP III antibody have the highest risk for developing SRC, but they seldom develop severe ILD. In Northern Europe and North America, approximately 60% of all patients experiencing SRC carry anti-RNAP III antibody. In anti-RNAP III-positive SSc patients, coexistence of anti-RNAP II antibody and a higher antibody titer measured by enzyme immunoassays are independently associated with the SRC risk. Lower incidence of SRC in Japan, French, and Mediterranean countries is thought to reflect the lower prevalence of anti-RNAP III antibody within these populations when compared with the UK and North America. Despite the

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lower prevalence of anti-RNAP III antibody in French SSc patients, this antibody remains the strongest serological marker for SRC. Anti-RNAP III antibody has been detected in patients complicating acute renal failure mimicking SRC in the absence of sclerotic skin disease, but these patients eventually develop rapidly progressive skin thickening. The presence of anti-RNAP III antibody should alert the managing physician to the increased risk of SRC and appropriate monitoring of blood pressure for early detection of this complication since the prompt introduction of angiotensin converting enzyme inhibitor can be life-saving. In recent cohorts, survival in patients with anti-RNAP III is better than those with anti-topo I, since SRC is more easily treated with angiotensin converting enzyme inhibitors than ILD. Finally, a case-control study using the large database identified anti-RNAP III antibody as one of risk factors for gastric antral vascular ectasia (GAVE; “watermelon stomach”).

It has been recently highlighted that there is an association with cancer among SSc patients with anti-RNAP III antibody in close temporal relationship to SSc onset. The prevalence of cancer was almost similar among SSc patients with anti-RNAP III, ACA, and anti-topo I, but the median disease duration at cancer diagnosis was quite different: 13.4, 11.1, and 1.2 years in anti-topo I-positive, ACA-positive, and anti-RNAP III-positive patients, respectively. Another large-scale cohort including 1,044 patients with SSc found anti-RNAP III is an independent marker of coincident cancer at SSc diagnosis. The temporal relationship between cancer and SSc among anti-RNAP III-positive patients suggests that SSc is a para-neoplastic disorder in this patient subset.

4. Anti-U3 RNP Antibody

Anti-U3 RNP antibody was first identified by RNA-IP assay as an autoantibody that precipitated the U3 RNP particle, which consists of 34-kD fibrillarin complexed with U3 RNA. This antibody produces bright nucleolar staining on IIF. Anti-U3 RNP antibody is found in 4–10% of patients with SSc, and is the most frequent in African Americans. Two thirds of the patients have dcSSc, but others are classified as typical lcSSc. Nearly one-third of the patients develop non-inflammatory skeletal myopathy, which is an SSc-related condition distinct from myositis overlap. It has been reported that severe GI involvement, including pseudo-obstruction and small bowel malabsorption, is associated with anti-U3 RNP antibody, especially in early course of the disease (<2 years). Severe internal organ involvement, including ILD, PAH, cardiomyopathy, and SRC are common in patients with anti-U3 RNP antibody, irrespective of dcSSc or lcSSc. Anti-U3 RNP antibody was identified as an independent risk factor for development of PAH in the large case-control study. In fact, PAH is the most common cause of death, leading to an increased mortality in this group of patients. An unusual combination of SRC followed later by PAH is occasionally found in patients with dcSSc and anti-U3 RNP antibody. Survival in anti-U3 RNP-positive patients is comparable to that in patients with anti-topo I antibody.

5. Anti-Th/To Antibody

Anti-Th/To antibody was identified by RNA-IP assay as an antibody that precipitated both 7-2 and 8-2 RNAs. Later, it has been shown that this antibody directs against subunits of mitochondrial RNA processing and ribonuclease P complexes. Of at least 6 subunits consisting of these complexes, a 120-kDa protein contains the major autoantibody epitope. Anti-Th/To antibody almost exclusively occurs in patients with lcSSc, although its frequency overall in SSc patients is only 2–5%. Like ACA-positive patients, anti-Th/To-positive patients are predominately Caucasians, but tend to have a shorter duration of Raynaud’s phenomenon before onset of other symptoms such as puffy fingers. Severe ischemic complications are infrequent, but this antibody is associated with significant ILD or PAH, which often occurs early in the disease course. In addition, patients with anti-Th/To can also develop pulmonary hypertension due to ILD. This increased frequency and severity of pulmonary complications result in a decreased survival compared with lcSSc patients without this antibody.

6. Anti-U11/U12 RNP Antibody

This autoantibody was identified by RNA-IP assay as the antibody that precipitated U11 RNA and U12 RNA together. It is a rare antibody specificity found in 1–3% of patients with SSc. This antibody produces speckled nuclear staining on IIF, and sometimes is associated with low-titer anti-U1 RNP antibodies. Patients with this antibody are classified as having either dcSSc or lcSSc. A characteristic feature of patients with anti-U11/U12 RNP antibody is a high frequency of ILD (>80%), which is often severe and rapidly progressive, and associated with a 2.25-fold greater risk of death in comparison with anti-U11/U12 RNP-negative patients with ILD.

7. Anti-PM-Scl Antibody

Anti-PM-Scl antibody was first identified by double immunodiffusion assay. The PM-Scl complex is composed of several subunits, of which the 100-kD and 75-kD proteins carry the main autoantigenic determinants.
Anti-PM-Scl antibody produces a homogenous nucleolar pattern, and is rarely found in non-Caucasian patients. Anti-PM-Scl-positive patients often present with the subacute myositis, but also have typical Raynaud’s phenomenon and scleroderma skin changes, usually lcSSc. They are most frequently diagnosed as having SSc-polymyositis (PM) overlap, but a significant proportion of the patients have rashes consistent with dermatomyositis (DM). This antibody is found in more than 25% of SSc patients with myositis overlap, but in only 2% of SSc patients overall. Serious internal organ involvement is rare, leading to a favorable prognosis. Myositis is usually mild and shows a good response to corticosteroids. In a recent large cross-sectional study of patients with idiopathic inflammatory myopathies, 9% had anti-PM-Scl antibody in the absence of any scleroderma skin changes.

8. Anti-Ku Antibody

Anti-Ku antibody was first identified by double immunodiffusion assay. The Ku autoantigen is now recognized as a heterodimer of 70-kD and 80-kD subunits. The prevalence of anti-Ku antibody in SSc patients is ~2%36. Anti-Ku antibody is primarily detected in patients with SSc in overlap. The majority of patients have typical Raynaud’s phenomenon and scleroderma skin changes, usually lcSSc. Concomitant inflammatory myopathy is common, but some have additional features of lupus. Anti-Ku antibody is occasionally detected in patients with systemic lupus erythematosus (SLE) without SSc features, but additional lupus-associated autoantibodies such as anti-DNA antibodies are always positive. Disease onset in anti-Ku-positive patients is usually younger than 40 years. Internal organ involvement is infrequent and usually mild if present, but arthritis is common. Anti-Ku antibody is associated with fewer vascular manifestations, such as DU or telangiectasia. Myositis is usually mild and shows a good response to corticosteroids, leading to a favorable prognosis.

9. Anti-RuvBL1/2 Antibody

This newly identified antibody recognizes a double hexamer consisting of RuvBL1 and RuvBL2, which is located in the nucleoplasm, by protein-IP assay. Anti-RuvBL1/2 antibody is a rare antibody specificity detected in 1–2% of patients with SSc. This antibody produces speckled nuclear staining with a high antibody titer on IIF. Patients with this antibody are mostly males and have a unique combination of clinical features, including dcSSc and myositis overlap. Internal organ involvement is mild in general, but some develop significant myocardial involvement.

10. Anti-U1 RNP Antibody

Anti-U1 RNP antibody was first identified by double immunodiffusion assay, in association with anti-Sm antibody, a lupus-specific ANA. Anti-U1 RNP antibodies are directed against the 70 K, A and C proteins associated with U1 RNA, while anti-Sm antibodies are directed against the B/B’ and D proteins that are core components of the U series small nuclear RNAs involved in pre-messenger RNA splicing (U1, U2, U4/U6, U5, and others). Anti-U1 RNP antibody, which produces a pure speckled pattern with a high antibody titer, is primarily detected in patients with SSc in overlap. This antibody is preferentially found in African Americans and Orientals. Anti-U1 RNP antibody was first described as a serologic marker for mixed connective tissue disease (MCTD), but is also found in sera from patients with SSc, SLE, PM/DM, or primary Sjögren’s syndrome. Disease onset is at a relatively young age with mid 30’s. SSc patients with this antibody usually present with inflammatory symptoms, such as myositis and arthritis. Raynaud’s phenomenon and puffy fingers occur early in the disease, but later these patients develop typical manifestations of SSc. Most of them have lcSSc, although approximately 20% develop dcSSc. Serious complications are relatively uncommon, but pulmonary complications, including PAH and ILD, are sometimes life-threatening. Prognosis is favorable in general, but PAH is the most common cause of death.

Screening and Detection of SSc-specific ANAs in Routine Clinical Practice

A conventional method for ANA detection is IIF on cultured HEp-2 cell slides. This technique is recommended as the autoantibody screening test because it is highly sensitive and provides additional information on the antibody titer and staining pattern. Speckled staining is often detected in patients with dcSSc and suggests the presence of anti-topo I in case of a high ANA titer (≥1 : 320) or anti-RNAP III in case of a low ANA titer (<1 : 160). Anti-U1 RNP and anti-RuvBL1/2 antibodies, which are associated with SSc in overlap, also produce a high-titer speckled pattern. A nucleolar pattern is fairly specific to SSc, and three major SSc-related anti-nucleolar antibodies are anti-U3 RNP, anti-Th/To, and anti-Pm-Scl antibodies. Anti-RNAP III antibody also produces a nucleolar pattern when anti-RNAP I antibody coexists, but a concomitant speckled staining is always present. A discrete speckled pattern is often detected in patients with lcSSc and suggests the presence of ACA.
Identification of individual SSc-specific ANAs requires additional techniques, such as double immunodiffusion assay and/or IP assay, but convenient immunoassays such as enzyme immunoassays (EIA) are widely used in routine clinical practice, because of simplicity, reproducibility, speed, and ability to handle many samples at the same time. The majority of commercially available immunoassays utilize recombinant autoantigens, which are expressed in the bacterial or eukaryotic system, while some assays still use native proteins purified from cellular extracts. It is necessary to use validated kits, since false-positive and false-negative results can occur. If the results obtained from EIA were inconsistent with clinical presentation, re-evaluation using original detection methods, such as IP assay, is highly recommended.

**Clinical Utility of SSc-specific ANAs**

1. **SSc Diagnosis**

SSc-specific ANAs are useful in diagnosis of SSc because of their highly specific nature. In fact, the presence of ACA, anti-topo I antibody, or anti-RNAP III antibody is included in one of the classification criteria for SSc, which has been proposed by the American College of Rheumatology (ACR) and the European League against Rheumatism (EULAR). In addition, the presence of SSc-specific ANA and nailfold capillary abnormalities are identified as independent predictors for future development of SSc in patients with Raynaud’s phenomenon, but without any features suggestive of connective tissue diseases, indicating that SSc-specific ANAs are also useful in identifying patients with pre-SSc efficiently.

2. **Disease Subsetting**

Classification of patients into dcSSc and lcSSc subsets is useful in predicting future organ involvement and prognosis, but more precise subgrouping is feasible by considering SSc-specific ANAs because of their strong correlations with main organ manifestations (Table 1). For example, three major dcSSc-specific ANAs, including anti-topo I, anti-RNAP III, and anti-U3 RNP antibodies, cover >80% of dcSSc patients, but major organ involvements linking to poor prognosis are apparently different. The presence of anti-topo I antibody in dcSSc patients provides independent prognostic information with regard to a risk for progression of ILD and a greater risk of mortality. In contrast, anti-RNAP III antibody has also been associated with increased mortality, primarily due to an increased prevalence of SRC, but with improvement in the management of SRC, such associations may not be replicated in the future. On the other hand, PAH still remains intractable condition with poor long-term survival even after introduction of a series of pulmonary vasodilators, and can occur in dcSSc patients with anti-U3 RNP antibody.

In addition, it is often difficult to classify patients into dcSSc or lcSSc early in the course of the disease when skin thickness is restricted to the distal portion of extremities. In this case, information on SSc-specific ANAs is useful in predicting the future extent of skin involvement. If one of dcSSc-associated ANAs, including anti-topo I, anti-RNAP III, and anti-U3 RNP antibodies, is present in circulation, disease modifying treatment should be indicated for preventing future organ damage at an early stage of dcSSc.

**Conclusions**

In summary, circulating ANA specificities are the best biomarkers for diagnosis and clinical subgrouping of SSc patients. Characterization of autoantibody status is an essential tool in clinical practice in the evaluation of patients who have SSc or are suspected to have this disease. In addition, because of their strong clinical associations, they allow clinicians to predict future disease manifestations and help to decide treatment strategy in individual patients. It is important to note, however, that clinical associations of autoantibodies are not absolute, and organ-specific manifestations can occur in the presence of any autoantibody reactivity.

**Conflict of Interest:** Kuwana M holds a patent on anti-RNAP III antibody-measuring kit.

**References**


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