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Rheumatology

Original Article

Clinical significance of anti-NOR90 antibodies in systemic sclerosis and idiopathic interstitial pneumonia

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Running head: Clinical significance of anti-NOR90 antibodies

Keywords: anti-NOR90 antibody, anti-nucleolar antibody, cancer, idiopathic interstitial pneumonia, interstitial lung disease, systemic autoimmune rheumatic disease, systemic sclerosis

Key messages:

● We developed an anti-NOR90 ELISA using a recombinant protein produced by *in vitro* transcription/translation.

● Anti-NOR90 antibodies can be a biomarker for idiopathic interstitial pneumonia with

characteristics of systemic sclerosis.

● Systemic sclerosis with anti-NOR90 antibodies can be complicated with interstitial lung disease and cancer.

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Abstract

Objective. Anti-NOR90 antibodies are usually found in patients with systemic sclerosis (SSc); however, their clinical relevance remains obscure. We developed an enzyme-linked immunosorbent assay (ELISA) for measuring them to investigate the clinical features of patients with anti-NOR90 antibodies.

Methods. Serum samples from 1,252 patients with various conditions from Nagoya University Hospital and 244 patients with idiopathic interstitial pneumonia (IIP) from Tosei General Hospital were included. Anti-NOR90 antibodies were assayed by an ELISA using the recombinant protein produced by *in vitro* transcription/translation.

Results. Five (0.4%) patients in the Nagoya University Hospital cohort had anti-NOR90 antibodies. One patient with diffuse cutaneous SSc, 3 with limited cutaneous SSc, and 1 with Raynaud's disease were positive for anti-NOR90 antibodies. Anti-NOR90 antibodies were found more frequently in patients with systemic scleroderma-spectrum disorders (SSDs) than without SSDs (5/316 vs. 0/936, $P<0.00101$) and were found more frequently in patients with SSc than without SSc (4/249 vs. 0/528, $P<0.0104$) in the systemic autoimmune rheumatic diseases cohort. Three of the 4 anti-NOR90-positive SSc patients had interstitial lung disease (ILD), and 2 of those 4 had cancer. Three (1.2%) patients in the Tosei General Hospital cohort had anti-NOR90 antibodies. All 3 of the anti-NOR90-positive IIP patients had gastrointestinal tract involvement, and 2 of those 3 had cancer or skin lesions observed in SSc.

Conclusions. Although anti-NOR90 antibodies are rarely found in clinics, our ELISA is useful for their detection. Further studies are needed to confirm the association of anti-NOR90 antibodies with ILD and cancer in SSc and IIP patients.

Introduction

A characteristic feature of patients with systemic autoimmune rheumatic diseases (SARDs) is the presence of serum autoantibodies which target intracellular components. Several systemic sclerosis (SSc)-related antibodies have been found [1, 2]. The anti-NOR90 antibody, which is a nucleolar type of anti-nuclear antibody (ANA), is also found in SSc patients. However, this antibody tends to be less specific to SSc and is reported in other SARDs, such as systemic lupus erythematosus (SLE), Sjögren's syndrome (SS) and rheumatoid arthritis (RA) [3], and in non-SARDs, such as hepatocellular carcinoma (HCC) [4]. For the detection of anti-NOR90 antibodies, immunoprecipitation (IPP) using recombinant protein produced by the cDNA of upstream binding transcription factor (UBTF)/NOR90, and Western blotting (WB) using HEp-2 cell nuclei [3] or MOLT-4 cell extracts have been performed [5]. Recently, a line blot assay (LB) for detecting anti-NOR90 antibodies has also been utilized [6].

Although some patients with interstitial lung disease (ILD) manifest autoimmune features, they do not always fulfill the diagnostic criteria for definite SARDs [7]. In the absence of definite SARDs, 10 to 20% of idiopathic interstitial pneumonia (IIP) patients have been reported to show serological abnormalities [7]. The most frequent clinical and serological markers for IIP patients with autoimmune features are Raynaud's phenomenon and ANA [7], but there are no explicit reports of patients with anti-NOR90 antibodies developing IIP.

In this study, we aimed to develop an enzyme-linked immunosorbent assay (ELISA) using the recombinant protein of NOR90 and to survey patients with various conditions for anti-NOR90 antibodies, including non-SARDs and IIP.

Methods

Serum samples

Serum samples were collected from the serum bank of the Department of Dermatology, Nagoya University Hospital between 1994 and 2020 (the Nagoya cohort). Other serum samples were collected from Department of Respiratory and Allergic Medicine, Tosei General Hospital between 2007 and 2015 (the Tosei cohort). Clinical information was collected retrospectively by reviewing the medical charts. Serum samples were collected at the first visit, with several exceptions, and were obtained from 1,252 consecutive patients. The median age at which the sera were obtained was 51 years (range: 3 to 88 years), and 35 (2.8%) juvenile patients (younger than 16 years old, which is the definition of SSc patient proposed by Zulian *et al.* [8]) were included. The ages of onset for the SSc patients were 12 to 82 years (median age: 54), and 4 juvenile patients were included. The numbers of patients in each disease category in this study are shown in Fig. 1. The 408 non-SARDs patients consisted of 35 with atopic disease, 33 with chronic fatigue syndrome, 23 with bullous pemphigoid, 17 with alopecia areata, 15 with Behcet's disease, and 285 with other conditions. Of the 249 sera with SSc (including overlap syndrome (OS) with SSc), 46 patients were excluded from the analysis of clinical features among all the SSc patients, diffuse cutaneous SSc (dcSSc) patients, and limited cutaneous (lcSSc) patients because of the unknown observation periods and the unavailability of information on those subsets. The remaining 203 patients were classified as 55 dcSSc and 148 lcSSc. The second cohort (the Tosei cohort) consisted of 244 patients with IIP. Their median age was 63 years (range: 21 to 76 years). Eleven anti-nucleolar antibody (ANoA)-positive IIP patients were excluded because serum samples from them were unavailable for this study.

As controls, serum samples from 26 healthy volunteers were used. The prototype serum with anti-NOR90 autoantibodies was a gift from Dr. Kuru, which showed ANoA in indirect immunofluorescence (IIF) and a positive result for anti-NOR90 antibodies by LB (Euroimmune, Lübeck, Germany). This study was conducted with the approval of the ethics committees of Nagoya University Hospital and Tosei General Hospital. All patients gave written consent of participation.

Criteria, entities and categories of diseases

SSc was diagnosed according to the classification of the 2013 classification criteria of the American College of Rheumatology (ACR) [9] and the ACR/European League Against Rheumatism (EULAR) [10]. SSc patients were classified as dcSSc or lcSSc according to the criteria of LeRoy *et al.* [11]. The age of onset for SSc patients was the age at which Raynaud's phenomenon and/or other SSc clinical features, such as ILD and gastrointestinal tract involvement (GI), first appeared. Chest computed tomography (CT) or chest X rays scans were performed to diagnose ILD. IIP was defined as interstitial pneumonia of unknown cause in which a patient did not fulfill the classification criteria for any specific SARDs or vasculitis, or whose lung disease was not caused by a drug or occupational-environmental exposure [12]. All 244 patients with IIP from the Tosei cohort were diagnosed by high-resolution CT (HRCT) and lung biopsy. The criteria of SARDs other than SSc and complications other than ILD in SSc are described in Supplementary File 1.

In this study, SARDs includes SSc, polymyositis (PM)/dermatomyositis (DM), SLE, SS, OS, RA, and mixed connective tissue disease (MCTD). Non-SARDs in the Nagoya cohort include IIP, cutaneous lupus erythematosus (CLE), localized scleroderma

(LS), Raynaud's disease (RD), Behcet's disease, polymyalgia rheumatica and various skin diseases. Patients with SARDs and without SARDs numbered 777 and 475, respectively. Systemic scleroderma-spectrum disorders (SSDs) consisted of SSc, OS with SSc, MCTD, RD, and LS. Patients with SSDs numbered 316.

Indirect immunofluorescence

All samples were analyzed with an IIF laboratory kit using HEp-2 cells (Fluoro HEPANA Test; MBL, Nagoya, Japan) [13].

Recombinant antigen of NOR90 for ELISA and immunoprecipitation

The full-length cDNA clone of UBTF (NM_014233.4)-harbouring the pBluescript II KS(+) plasmid (product ID: F0235) was purchased from GenPlus® ORF Clone (Genscript, Tokyo, Japan). Biotinylated recombinant proteins produced by *in vitro* translation and transcription (TnT) were produced from the cDNA, using the T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA) according to our published protocol [14]. Briefly, 800 µl of transcription and translation Quick Master Mix, 20 µl of 1 mM methionine, 30 µl of transcend biotin-lysyl-tRNA, 120 µl of water and 30 µl of DNA (1 µg/µl) were mixed and then incubated at 30℃ for 60 minutes.

ELISA

Anti-NOR90 antibodies were quantitatively tested by antigen-capture ELISA according to our published protocols [15]. A 96-well Nunc™ Immobilizer™ Streptavidin Plate (Thermo Scientific Nunc, Roskilde, Denmark) was incubated with 1 µl/well of *in vitro* TnT reaction mixture including biotinylated recombinant protein. The wells were

incubated with 1:1,000 diluted sera and probed with anti-human IgG antibody conjugated with horseradish peroxidase (HRP) (Dako, Glostrup, Denmark) (1:30,000 dilution). After incubation with SuperSignal® ELISA Femto Maximum Sensitivity Substrate (Thermo Scientific Pierce, Rockfold, IL, USA), the relative luminescence unit (RLU) was determined using the GloMax®-Multi Detection System (Promega). Each sample was tested in duplicate, and the mean RLU with the background subtracted was used for data analysis. The RLU of the samples was converted into units using a standard curve created by a prototype positive serum. As a standard, the prototype serum diluted 1:5 serially starting from 1:100 was run. Units correlated with the titers of antibodies: 1:100 dilution, 625 units; 1:500 dilution, 125 units; 1:2,500 dilution, 25 units; 1:12,500 dilution, 5 units; 1:612,500 dilution, 1 unit; 1:3,062,500 dilution, 0.2 unit. Preliminary experiments on an ELISA that included the prototype serum showed antigen concentrations of 1 µL of TnT mixture/well and a serum sample dilution of 1:1,000 to be suitable for the assay. The cutoff value was set to 0.28 units from the mean units obtained from 26 control sera of healthy volunteers +5 standard deviations (SD) according to our previous study [15].

Immunoprecipitation

IPP was performed using *in vitro* TnT products as previously described [14], with slight modifications. In short, 5 µl of patient sera was mixed and incubated with 10 µl of a 50% slurry of Protein G Sepharose (PG) (GE Healthcare, Buckinghamshire, UK) and 10 µl of the *in vitro* TnT products of UBTF in 300 µl of 0.5 M NaCl, 2 mM EDTA, 50 mM Tris, pH 7.5, 0.3% IGEPAL CA-630 (Nonidet P40) (NET/NP40) buffer at 4℃ for 60 minutes. After being washed 5 times with IPP buffer, the antibody-antigen complexes retained by the PG were mixed with 10 µl of 2x sodium dodecyl sulfate (SDS)

sample buffer, boiled at 65℃ for 10 minutes, fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto an Immobilon-P transfer membrane (Millipore Corp., Billerica, MA, USA). The biotinylated proteins were then detected with Western Blue Substrate (Promega).

Western Blotting

WB was performed using K562 cell nuclear extracts (Santa Cruz Biotechnology, Dallas, TX, USA) as described previously [13], with minor modifications. Extracts mixed with SDS sample buffer were subjected to SDS-PAGE and transferred onto an Immobilon-P transfer membrane. After treatment with blocking buffer (5% skim milk in Tween-PBS), the membranes were incubated with diluted serum samples (1:100) in the above blocking buffer for 60 minutes, washed in Tween-PBS three times for 30 minutes, and incubated in HRP-conjugated anti-human IgG (1/1,000 dilution in the above blocking buffer) (Dako) for 60 minutes. After washing in Tween-PBS three times for 30 minutes, color development was carried out with EzWestBlue (ATTO Corp., Tokyo, Japan).

Detection of other autoantibodies

Autoantibodies other than anti-NOR90 antibodies were detected by the following methods. Anti-RNA polymerase III antibodies were analyzed by ELISA (MESACUP-anti-RNA polymerase III Test; MBL, Nagoya, Japan). Anti-Scl70 antibodies were analyzed by ELISA (MESACUP™-3 Test Scl-70; MBL, Nagoya, Japan). Anti-PM/Scl antibodies were investigated by our previously reported methods [16]. Anti-Th/To antibodies and anti-U3RNP antibodies were investigated by our in-house ELISA and IPP using the recombinant proteins produced by *in vitro* transcription/translation,

respectively (manuscript in preparation).

Statistical analyses

Data were statistically evaluated using SPSS Statistics (IBM, Tokyo, Japan). Fisher's exact tests and unpaired t-tests were used for comparison of frequencies. *P* values of less than 0.05 were considered significant.

Results

Diseases and numbers of patients with anti-nucleolar antibody

We selected ANoA-positive sera to further investigate the presence of anti-NOR90 antibodies. The numbers of ANoA-positive patients for each disease are shown in brackets in Fig. 1. In the Nagoya cohort, the ANoA-positive patients with SARDs and without SARDs numbered 86 (6.9%) and 42 (3.4%), respectively. ANoA-positive patients with SSDs numbered 53 (4.2%). For the Tosei cohort, 24 (9.8%) patients with IIP had ANoA; however, 11 of those 24 were excluded due to the unavailability of their sera for study. The remaining 13 (5.3%) patients consisted of 8 men and 5 women, and their median age was 69 years (range: 52 to 74 years).

ELISA and IPP using recombinant protein for anti-NOR90

For the mass-screening of anti-NOR90 antibodies, we aimed to develop an ELISA using biotinylated recombinant UBTF/NOR90.

128 ANoA-positive sera from the Nagoya cohort and 13 ANoA-positive sera from the Tosei cohort were examined for anti-NOR90 antibodies by our in-house ELISA (Fig. 2). Five sera from the Nagoya cohort (Fig. 2, Patients 1 to 5) and 3 sera from the Tosei cohort (Fig. 2, Patients 6 to 8) were positive for anti-NOR90 antibodies. These 8 sera were further investigated by immunoprecipitation using the recombinant protein to confirm the ELISA results (Fig. 3A, lanes 1 to 8) in addition to 2 controls [serum with the highest unit in the ELISA-negative group (Fig. 3A, lane 9) and serum from a normal individual (Fig. 3A, lane 10)]. All 8 ELISA-positive sera immunoprecipitated the biotinylated recombinant NOR90, whereas the sera from the 2 controls did not.

Western blotting using K562 cell extract

All 8 ELISA/IPP-positive sera showed reactivity with a 90-kDa doublet band (Fig. 3B, lanes 1 to 8), a pattern that was reported by previous anti-NOR90 WB analysis [3, 5], whereas neither of the 2 controls showed reactivity. According to these results, all 8 ELISA-positive sera were judged to have antibodies against NOR90.

Frequencies of the anti-NOR90 antibody in systemic sclerosis and its spectrum disorders

The frequencies of anti-NOR90 antibodies in the Nagoya cohort were analyzed (Table 1). Five of the 128 (3.9%) ANoA-positive patients had anti-NOR90 antibodies. No significant difference in anti-NOR90 antibody prevalence was found between the patients with SARDs and without SARDs. However, the positive rate of anti-NOR90 antibodies was significantly higher in patients with SSDs than in those without SSDs in all patients (5/316 vs. 0/936, $P<0.00101$) (Table 1). The positive rate of anti-NOR90 antibodies was also significantly higher in patients with SSc than in those without SSc among SARDs for all patients (4/249 vs. 0/528, $P<0.0104$) (Table 1). However, the positive rate of anti-NOR90 antibodies was not significantly higher in patients with SSc than in those without SSc among SARDs for the ANoA-positive patients only. Of the 46 patients with SSc who were excluded due to the unavailability of detailed information, 6 had ANoA; however, none of those 6 had anti-NOR90 antibodies. There was no significant relationship between the presence of anti-NOR90 antibodies and the patient's age in the Nagoya cohort ($P<0.129$ by unpaired t-tests) or the Tosei cohort ($P<0.669$). Similarly, no significant difference was found between anti-NOR90 antibodies and the ages of onset for the SSc patients ($P<0.494$).

Clinical significance of anti-NOR90 antibody in systemic sclerosis

The clinical characteristics of 8 patients with anti-NOR90 antibodies are summarized in Table 2. For all 8 anti-NOR90-positive sera, the autoantibody pattern in IIF was the punctate nucleolar pattern (ICAP AC-10), based on the International Consensus on Autoantibody Patterns (ICAP: https://anapatterns.org/trees-ful.php [17]). Patient 1 also had anti-RNA polymerase III antibodies, but none of the anti-NOR90-positive patients had any other SSc-marker ANoAs, such as anti-PM/Scl, anti-Th/To, or anti-U3RNP antibodies (data not shown). Of the 5 patients with anti-NOR90 antibodies from the Nagoya cohort (Table 2, Patients 1 to 5), 3 (75%) and 2 (50%) anti-NOR90-positive SSc patients had ILD and cancer, respectively.

We investigated the relationship between anti-NOR90 antibodies, SSc subsets, and complications in the 203 patients with SSc (Table 3). A similar analysis was also performed for individual SSc subsets: dcSSc (Supplementary Table S1) and lcSSc (Supplementary Table S2). There were no items with statistically significant differences between anti-NOR90-positive and -negative patients. However, in the lcSSc patients, ILD (2/3 vs. 37/144, $P<0.172$) and cancer (2/3 vs. 28/145, $P<0.105$) were relatively frequently detected in the anti-NOR90-positive patients.

Clinical features of anti-NOR90 antibody-positive idiopathic interstitial pneumonia patients

In the 13 ANoA-positive patients with IIP from the Tosei cohort, 3 patients (3/13, 23.1%) had anti-NOR90 antibodies (Table 2, Patients 6 to 8). All 3 anti-NOR90-positive patients had GI. None of the 3 anti-NOR90-positive patients fulfilled the criteria for SSc.

There were no statistically significant differences in the prevalence of cancer between IIP patients with anti-NOR90 antibodies and those without anti-NOR90 antibodies (2/3 vs. 35/230, $P<0.0665$); however, the anti-NOR90-positive IIP patients tended to have an elevated risk of cancer as a complication.

Discussion

In 1987, antibodies to NOR90 in 4 scleroderma patients were described for the first time [18]. Since then, accumulated cases have been reported, and it is known that anti-NOR90 antibodies are not always specific to SSc. Fujii *et al.* showed that 7 (7.7%) patients with SS, 3 (3.3%) with RA, 2 (2.2%) with SSc, and 1 (1.1%) with OS (SSc+RA) had anti-NOR90 antibodies [19]. It should be noted that 4 of the 7 SS patients had secondary SS, not primary SS [19]. Imai *et al.* showed that anti-NOR90 antibodies were present in 2 patients with SLE, 2 with RD, 1 with RA, and 3 with other diseases [20]. However, their study did not mention the patient population for each disease.

In the present study, anti-NOR90 antibodies were present significantly more frequently in patients with SSDs than in patients without SSDs, and significantly more frequently in patients with SSc than in patients with other SARDs. Thus, anti-NOR90 antibodies could be a good biomarker for suspected SSc in patients with SARDs. A limitation of this study is that the results could have been affected by selection bias, given that the SSc patients were the most numerous group. The present study indicates that 3 of the 4 anti-NOR90-positive SSc patients had ILD and 2 of the 3 anti-NOR90-positive IIP patients had skin symptoms of SSc. Systemic sclerosis sine scleroderma (ssSSc), first described by Rodnan and Fennel [21], is characterized by the total or partial absence of cutaneous manifestations of SSc with the occurrence of internal organ involvement and serological abnormality. Although there have been no definite criteria for ssSSc, Poomoghim *et al.* reviewed the literature and reported that all 19 ssSSc patients had GI and 13 ssSSc patients had pulmonary involvement (ILD + pulmonary hypertension) [22]. While anti-NOR90-positive IIP patients in this study did not fulfill the diagnostic criteria for SSc, they had the characteristics of ssSSc. Anti-NOR90 antibodies might be

suggestive of SSc and IIP overlap. It is significant to measure the anti-NOR90 antibodies in ANoA-positive IIP patients. Since all our anti-NOR90-positive sera gave the punctate nucleolar pattern in IIF, anti-NOR90 antibodies can be screened by the IIF pattern and further confirmed by other assays, such as WB, LB, and our ELISA.

The incidence of cancer in SSc patients is known to be elevated compared with the general population [23]. SSc patients, especially those with anti-RNA polymerase III antibodies, are known to be more likely to develop cancer. Mecoli *et al.* showed that anti-Th/To antibodies might have a protective effect against cancer [24]. However, the association between anti-NOR90 antibodies and cancer has not been described. In the present study, cancer comorbidity in the anti-NOR90-positive patients was high (4/8, 50%). Interestingly, UBTF/NOR90 is also a target oncogenic signaling and is overexpressed in HCC (reviewed in [25]).

In Patient #1, high-titer (≧1:320) IIF staining with the speckled pattern was seen and the staining may correspond to anti-RNA polymerase III antibodies. Anti-RNA polymerase III antibodies are known to be associated with diffuse cutaneous involvement [2], whereas anti-NOR90 antibodies are probably associated with lcSSc [3]. Her clinical subset, dcSSc, might be influenced by the coexistence of anti-RNA polymerase III antibodies.

LB has been recently and widely used as a method for detecting autoantibodies. However, Hamaguchi *et al*. revealed that 6 of the 9 samples (66.7%) at the low cutoff value and 2 of the 5 samples (40%) at the high cutoff value were anti-NOR90-positive with LB and negative with IPP [26]. Our newly developed anti-NOR90 ELISA should be validated for sensitivity and specificity in large cohorts.

There are several limitations to this study. It was retrospective, with observation

periods that were short in some cases. Very different numbers of patients with each disease were investigated, and there were only several serum samples with anti-NOR90 antibodies. For IIP, 11 of the 24 ANoA-positive IIP patients were excluded due to the unavailability of sera and their serum samples could not be investigated for anti-NOR90 antibodies. Furthermore, only ANoA-positive serum samples were measured for anti-NOR90 antibodies, since ANoA-negative sera with anti-NOR90 antibodies have not been reported. Indeed, we have investigated more than 260 autoimmune serum samples without ANoA using our in-house anti-NOR90 ELISA in our laboratory, but no anti-NOR90-positive sera have been found (data not shown).

One large-scale study on anti-NOR90 antibodies was conducted on German patients with various conditions [27]. In that study, 9 of the 26,631 patients were clarified to have anti-NOR90 antibodies by IIF using Hep-2 cells, a chromosome spread assay, and WB using HeLa-S3 nucleolar extract. Our study is the first to show the clinical features of anti-NOR90 antibodies in a cohort of more than 1,000 Japanese patients. Further research with a greater number of sera with anti-NOR90 antibodies is needed to reveal the clinical characteristics of patients with anti-NOR90 antibodies.

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Figure legends

FIG. 1 Disease classification of patients included in the present study.

Numbers in brackets for each disease indicate the number of ANoA-positive patients. Green boxes indicate SARDs. Yellow boxes indicate non-SARDs. Red letters indicate systemic scleroderma-spectrum disorders. In the analysis of the relationship between anti-NOR90 antibodies and complications in SSc, 46 SSc patients were excluded. Eleven ANoA-positive IIP patients were also excluded (see the text).

CLE, cutaneous lupus erythematosus; dcSSc, diffuse cutaneous systemic sclerosis; DM, dermatomyositis; IIP, idiopathic interstitial pneumonia; lcSSc, limited cutaneous systemic sclerosis; MCTD, mixed connective tissue disease; OS, overlap syndrome; PM, polymyositis; RA, rheumatoid arthritis; SS, Sjögren's syndrome; SLE, systemic lupus erythematosus; SSc, systemic sclerosis

FIG. 2 Anti-NOR90 antibodies in 141 ANoA-positive serum samples and 26 healthy control subjects measured by ELISA.

We used 1 μL/well of TnT mixture and patient serum samples diluted to 1:1,000 to measure each samples. Antibody units were calculated from the RLU using a standard curve obtained from serial concentrations of a prototype serum sample with anti-NOR90

antibodies. The broken line indicates the cutoff value (0.28 units). Closed circles Pt.1 to 8 correspond to the patients shown in Table 2.

IIP, idiopathic interstitial pneumonia; Pt., patient; RD, Raynaud's disease; SSc, systemic sclerosis

FIG. 3 IPP of recombinant UBTF/NOR90 with patients' sera and WB using K562 extract for anti-NOR90 antibodies.

Anti-NOR90 antibodies were detected by IPP with biotinylated recombinant UBTF/NOR90 protein (**A**) and WB using K562 nuclear extract (**B**). Lane In. shows an input lane which contains half the dose of biotinylated recombinant protein used for IPP. The serum of lane P is a prototype serum with anti-NOR90 antibodies. Lanes 1 to 8 correspond to the anti-NOR90 antibody-positive patients shown in Table 2. Lane 9 is the highest-titer serum among the ELSA-negative sera. Lane 10 is serum from a healthy control. An arrowhead and an asterisk denote the position of 90 kDa in IPP and WB analysis, respectively.

TABLE 1. Frequencies of anti-NOR90 antibodies in SARD, SSD and SSc patients

	number of anti-NOR90 (+) patients in all samples	*P*	number of anti-NOR90 (+) patients in ANoA (+) samples	*P*
SARDs vs. non-SARDs	4/777 vs. 1[a]/475	<0.656	4/86 vs. 1[a]/42	1
SSDs vs. non-SSDs	5/316 vs. 0/936	<0.00101	5/53 vs. 0/75	<0.0109
SSc vs. non-SSc in SARDs	4/249 vs. 0/528	<0.0104	4/43 vs. 0/43	<0.117

In this study, SARDs consist of SSc, PM/DM, SLE, SS, OS, RA, and MCTD. SSDs consist of SSc, OS with SSc, MCTD, RD, and LS. RD and LS, which are scleroderma-spectrum disorders, are included in non-SARDs. [a] One patient with RD. ANoA, anti-nucleolar antibody; DM, dermatomyositis; LS, localized scleroderma; MCTD, mixed connective tissue disease; OS, overlap syndrome; PM, polymyositis; RA, rheumatoid arthritis; RD, Raynaud's disease; SARDs, systemic autoimmune rheumatic diseases; SS, Sjögren's syndrome; SLE, systemic lupus erythematosus; SSc, systemic sclerosis; SSDs, systemic scleroderma-spectrum disorders.

TABLE 2. Clinical features of patients with anti-NOR90 antibodies

Pt	Diagnosis	Age	Sex	IIF pattern, titer	Other SSc-related antibodies	Skin symptoms	ILD	Cancer	PAH	SRC	GI	Score[a]	Observation period (years)
1	dcSSc	71	F	No., 1:2560 Spe., 1:1280	Pol-III	sclerodactyly, telangiectasia	+	-	+	-	+	20	2
2	lcSSc	73	F	No., 1:1280	N.P.	sclerodactyly, ANC	+	stomach, colon	-	-	-	17	10.7
3	lcSSc	70	M	No., 1:640	N.P.	sclerodactyly, ANC	+	lung	-	-	-	17	0.9
4	OS (lcSSc, RA)	45	F	No., 1:1280 Spe., 1:320	N.P.	sclerodactyly, RP, PF	-	-	-	-	+	16	17
5	Raynaud's disease	8	F	No., 1:640 Spe., 1:160	N.P.	RP, PF, ANC	-	-	-	-	-	7	8
6	IIP	73	F	No., 1:640 Spe., 1:160	N.P.	RP, PF	+	Lung	-	-	+	7	12
7	IIP	60	F	No., 1:320	N.P.	ANC	+	-	-	-	+	4	10
8	IIP	70	F	No., 1:320	N.P.	N.P.	+	DLBCL	+	-	+	2	3

[a] Total score of the ACR/EULAR criteria for the classification of SSc. ANC, abnormal nailfold capillaries; dcSSc, diffuse cutaneous systemic sclerosis; DLBCL, diffuse large B-cell lymphoma; GI, gastrointestinal tract involvement; IIF, indirect immunofluorescence; IIP, idiopathic interstitial pneumonia; ILD, interstitial lung disease; lcSSc, limited cutaneous systemic sclerosis; No., nucleolar pattern; N. P., not present; OS, overlap syndrome; PAH, pulmonary arterial hypertension; PF, puffy fingers; Pol-III, anti-RNA polymerase III antibody; Pt, patient; RA, rheumatoid arthritis; RP, Raynaud's phenomenon; SRC, scleroderma renal crisis; Spe., speckled pattern.

TABLE 3. Presence/absence of anti-NOR90 antibodies and clinical characteristics in SSc patients

	SSc	Anti-NOR90 antibodies (+)	Anti-NOR90 antibodies (-)	*P*
Numbers	203	4	199	
Females/males	184/19	3/1	181/18	<0.328
Ages, range (median)	16 - 84 y. o. (58 y. o.)	45 - 73 y. o. (70.5 y. o.)	16 - 84 y. o. (58 y. o.)	<0.917
Observation periods, range (median)	0.1 - 46 yrs. (11.1 yrs.)	0.9 - 17 yrs. (6.3 yrs.)	0.1 to 46 yrs. (11.4 yrs.)	<0.643
ILD (%)	78 (38.6)[a]	3 (75)	75 (37.9)[a]	<0.301
Cancer (%)	36 (17.7)	2 (50)	34 (17.1)	<0.146
PAH (%)	22 (12.2)[b]	1 (25)	21 (11.9)[b]	<0.408
SRC (%)	7 (3.4)	0 (0)	7 (3.5)	1
GI (%)	129 (63.5)	2 (50)	127 (63.8)	<0.624

[a] One case was excluded because detailed information was unavailable. [b] Twenty-two cases were excluded because detailed information was unavailable. GI, gastrointestinal tract involvement; ILD, interstitial lung disease; PAH, pulmonary arterial hypertension; SRC, scleroderma renal crisis; SSc, systemic sclerosis; y. o., years old.; yrs., years.

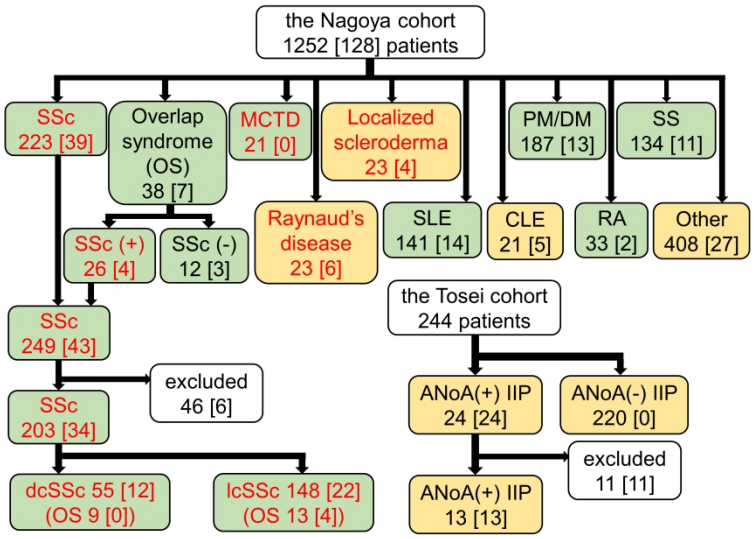

Figure 1 Disease classification of patients included in the present study.

338x190mm (300 x 300 DPI)

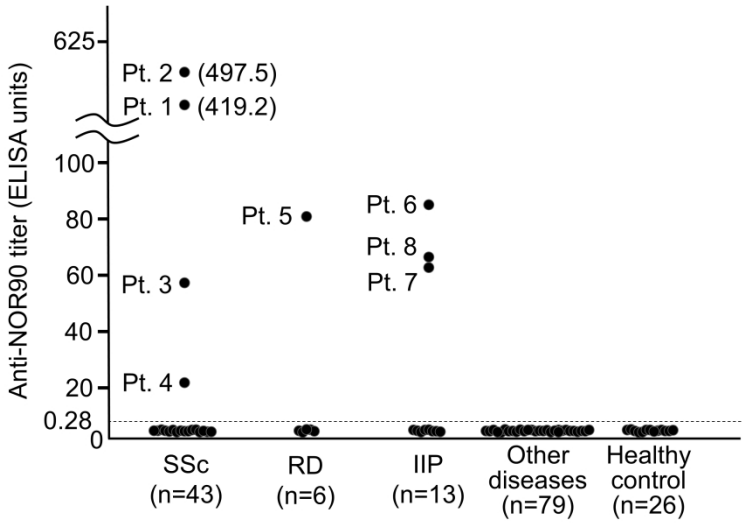

Figure 2 Anti-NOR90 antibodies in 141 ANoA-positive serum samples and 26 healthy control subjects measured by ELISA.

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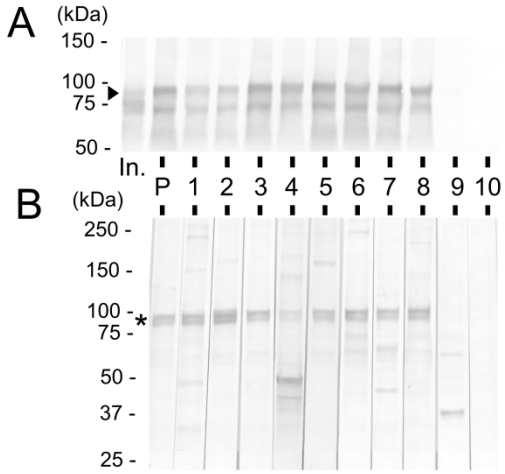

Figure 3 IPP of recombinant UBTF/NOR90 with patients' sera and WB using K562 extract for anti-NOR90 antibodies.

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Supplementary Information

Supplementary File 1. Supplementary methods

Supplementary Table S1. Anti-NOR90 antibodies and complications in dcSSc patients

Supplementary Table S2. Anti-NOR90 antibodies and complications in lcSSc patients

Supplementary File 1.

Supplementary methods

Criteria, entities and categories other than SSc, ILD, and IIP

The dermatomyositis (DM) and polymyositis (PM) patients fulfilled Bohan and Peter's criteria [1], except for clinically amyopathic DM (CADM), which was defined by Sontheimer's criteria [2]. The criteria proposed by the Juvenile Dermatomyositis Working Party (Network for JDM) of the Pediatric Rheumatology European Society in 2006 were used for the diagnosis of juvenile DM (JDM) [3]. SLE was defined in accordance with the 1997 revised ACR SLE classification criteria [4]. The diagnosis and classification of discoid lupus erythematosus (DLE) and subacute cutaneous lupus erythematosus (SCLE) were based on clinical and histological characteristics and on serological parameters according to the Dusseldorf Classification 2004 [5]. The classification criteria for SS proposed by the American-European Consensus Group were used to diagnose primary SS [6]. Overlap syndrome (OS) was diagnosed as cases that fulfilled the criteria for two SARDs. RA was diagnosed by the 2010 classification criteria [7]. The criteria for Raynaud's disease (RD) include symmetric attacks, the absence of tissue necrosis, ulceration or gangrene, and the absence of a secondary

cause including a SARD [8]. Localized scleroderma (LS) was diagnosed based on the definition of a disease of the skin and subcutaneous tissue that leads to patches of thickened skin whose biopsy reveals dermal fibrosis similar to the histopathological changes seen in the thickened skin of SSc [9]. Mixed connective tissue disease (MCTD) was defined by the 2019 diagnostic criteria for MCTD of the Japan Research Committee of the Ministry of Health, Labor and Welfare for systemic autoimmune diseases [10].

Complications of SSc

Scleroderma renal crisis (SRC) was defined by the occurrence of malignant arterial hypertension or rapidly progressive oliguric renal failure without other discernible causes during the course of SSc [11]. Gastrointestinal tract involvement (GI) was defined as gastroesophageal reflux disease (GERD), dysphagia, bacterial overgrowth requiring antibiotics, and/or paralytic ileus [12]. Pulmonary artery hypertension (PAH) was diagnosed from a mean pulmonary arterial pressure of more than 20 mmHg measured directly by right heart catheterization according to the 6th World Symposium on Pulmonary Hypertension (WSPH) in 2018 [13], or peak tricuspid regurgitation velocity of more than 3.4 m/s by Doppler echocardiography [14].

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Supplementary TABLE S1. Anti-NOR90 antibodies and complications in dcSSc patients

	dcSSc	Anti-NOR90 antibodies (+)	Anti-NOR90 antibodies (-)	*P*
Number	55	1	54	
Female/male	47/8	1/0	46/8	1
Age range (median)	22 - 82 y.o. (57 y.o.)	71 y.o. (71 y.o.)	22 - 82 y.o. (57 y.o.)	<0.233
Observation period range (median)	0.1 - 46 yrs. (10 yrs.)	2 yrs. (2 yrs.)	0.1 - 46 yrs. (10.5 yrs.)	<0.323
ILD (%)	39 (70.9)	1 (100)	38 (70.4)	1
Cancer (%)	6 (10.9)	0 (0)	6 (11.1)	1
PAH (%)	9 (18.4)[a]	1 (100)	8 (16.7)[a]	1
SRC (%)	4 (7.3)	0 (0)	4 (7.4)	1
GI (%)	34 (61.8)	1 (100)	33 (61.1)	1

[a] Six cases were excluded because detailed information was unavailable. dcSSc, diffuse cutaneous systemic sclerosis; GI, gastrointestinal tract involvement; ILD, interstitial lung disease; PAH, pulmonary arterial hypertension; SRC, scleroderma renal crisis; y.o., years old.; yrs., years.

Supplementary TABLE S2. Anti-NOR90 antibodies and complications in lcSSc patients

	lcSSc	Anti-NOR90 antibodies (+)	Anti-NOR90 antibodies (-)	*P*
Number	148	3	145	
Female/male	137/11	2/1	135/10	<0.209
Age range (median)	16 - 84 y.o. (58 y.o.)	45 - 73 y.o. (70 y.o.)	16 - 84 y.o. (58 y.o.)	<0.582
Observation period range (median)	0.1 - 44 yrs. (11.7 yrs.)	0.9 - 17 yrs. (10.7 yrs.)	0.1 - 44 yrs. (11.8 yrs.)	<0.492
ILD (%)	39 (26.5) [a]	2 (66.7)	37 (25.7)[a]	<0.172
Cancer (%)	30 (20.3)	2 (66.7)	28 (19.3)	<0.105
PAH (%)	13 (9.8)[b]	0 (0)	13 (10.1)[b]	1
SRC (%)	3 (2.0)	0 (0)	3 (2.1)	1
GI (%)	95 (64.2)	1 (33.3)	94 (64.8)	<0.292

[a] One case was excluded because detailed information was unavailable. [b] Sixteen cases were excluded because detailed information was unavailable. GI, gastrointestinal tract involvement; ILD, interstitial lung disease; lcSSc, limited cutaneous systemic sclerosis; PAH, pulmonary arterial hypertension; SRC, scleroderma renal crisis; y.o., years old.; yrs., years.