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ORIGINAL ARTICLE

Makoto Goto · Masako Okawa-Takatsuji
Shinichi Aotsuka · Hidenori Nakai · Masatosi Shimizu
Hideyuki Goto · Akira Shimamoto · Yasuhiro Furuichi

Significant elevation of IgG anti-WRN (RecQ3 RNA/DNA helicase) antibody in systemic sclerosis

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Abstract Werner syndrome, caused by the homologous mutation of RecQ3 RNA/DNA helicase (WRN), is often misdiagnosed as systemic sclerosis (SSc) because of apparent similar skin changes and its relatively high frequency in Japan. The present study was undertaken to determine whether anti-WRN antibodies assayed by specific enzyme-linked immunosorbent assay occur in 41 SSc patients (30 diffuse and 11 limited types) and, if so, to determine any clinical association, such as skin sclerosis. Serum level of IgG anti-WRN antibody in SSc was significantly higher than that from 30 age- and sex-matched normal volunteers ($P < 0.001$). The serum level of IgG anti-WRN antibody in diffuse type SSc was significantly higher than the limited type ($P < 0.05$). A significant correlation was observed between serum levels of IgG anti-topoisomerase I antibody and IgG anti-WRN antibody in the same samples from SSc ($P < 0.05$). Moreover, in 119 normal healthy individuals aged from 0 to 99 years, a statistically significant correlation ($P < 0.001$) existed between serum level of IgG anti-WRN antibody and advancing age. A significantly higher level of IgG autoantibody specific for WRN detected in diffuse than in limited type SSc and normal may contribute to the pathogenesis of skin sclerosis in SSc.

M. Goto (✉)
Division of Anti-Ageing and Longevity Sciences, Department of Clinical Engineering, Faculty of BioMedical Engineering, Toin University of Yokohama, 1614 Kurogane-cho, Aoba-ku, Yokohama 225-8502, Japan
Tel./Fax +81-45-974-5132
e-mail: goto@cc.toin.ac.jp

M. Okawa-Takatsuji · S. Aotsuka
Department of Clinical Immunology, International Medical Center of Japan, Tokyo, Japan

H. Nakai
Department of Internal Medicine, Kinnikyo Sapporo Hospital, Sapporo, Japan

M. Shimizu
Division of Rheumatology, Hino Hospital, Hino, Japan

H. Goto · A. Shimamoto · Y. Furuichi
GeneCare Research Institute, Kamakura, Japan

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Introduction

Systemic skin sclerosis is the hallmark of both systemic sclerosis (SSc) and Werner syndrome (WS; MIM #27770). Werner syndrome, caused by the homologous mutation of RecQ3 RNA/DNA helicase (WRN), is an autosomal recessively inherited disease with progeroid phenotypes.[1–5] Patients with WS manifest a series of accelerated forms of normal aging phenotypes after an early termination of teenage growth spurt followed by gray hair, alopecia, juvenile cataract, voice change, skin atrophy, skin sclerosis, telangiectasia, skin hyper-/hypopigmentation, skin ulcers, osteoporosis, hypogonadism, diabetes mellitus, atherosclerosis, subcutaneous calcification, brain atrophy, and malignant tumors, followed by death before 50 years of age.[1,2] They also manifest a mild lung fibrosis, sclerodactyly, and in some limited cases sicca syndrome, thyroid diseases, arthritis, and systemic lupus erythematosus, but have never shown an esophageal dilatation, Raynaud phenomenon, or pulmonary hypertension.[1,6] As a whole, patients with WS mimic an accelerated form of natural aging and thus WS has been selected as a top-ranking model of human aging.

Helicases are RNA/DNA metabolism enzymes with the common function of unwinding of the helical structure of double-stranded RNA/DNA to single strands.[7] RecQ3 RNA/DNA helicase (WRN) was believed to function in the nucleolus within the nucleus.[8,9] The DNA enzyme; topoisomerase I, whose antibody (anti-topoisomerase I: Scl-70) is the specific disease marker antibody for SSc, colocalizes and seems to work in association with RecQ3 RNA/DNA helicase in cases of RNA/DNA metabolism such as repair, replication, recombination, and transcription.[10–12] The nucleolar pattern of fluorescent antinuclear antibody was usually detected in the sera from SSc.[12] As the frequency of WS in Japan is extremely high compared with elsewhere[1,5] as well as clinically similar skin changes such as

Table 1. Demography of the patients with systemic sclerosis

		Diffuse	Limited	Normal volunteer
No. of patients		30 (F 27, M 3)	11 (F 11)	30 (F 28, M 2)
Age (years)	Mean ± SD	47.7 ± 13.8	51.7 ± 17.0	48.2 ± 15.3
	Range	23–72	17–72	24–70
Disease duration (years)	Mean ± SD	11.4 ± 8.6	8.6 ± 8.1	–
	Range	1–32	2–30	–
Therapy (%)	Prednisolone (<10 mg)	43.3	36.4	–
	D-Penicillamine	26.7	36.4	–
	PGI_2	10	0	–
	Calcium channel blocker	7	9.1	–
	None	20	18.1	–

PGI_2, prostacyclin

scleroderma, hyper/hypopigmentation, telangiectasia, skin ulcer, and subcutaneous calcification between WS and SSc, we studied the possible contribution of anti-WRN autoantobodies to the pathogenesis of skin sclerosis in SSc.

Patients and methods

Patients

Sera from a total of 41 patients with SSc who met the American College of Rheumatology (formerly, American Rheumatism Association) preliminary classification criteria for SSc[13] and 119 apparently healthy normal individuals of both sexes aged from 0 to 99 years were collected and stored at −70°C until use. In comparing the difference of antibody levels between SSc and normal, 30 age- and sex-matched healthy normal volunteers among the healthy individuals were selected as the control. The demographic data of the patients are summarized in Table 1. Basically, no significant difference was found between the patients with diffuse type SSc and limited type SSc in terms of age, disease duration, and type of therapy. The Institutional Review Board of Toin University, Yokohama approved the study.

Preparation of RecQ3 RNA/DNA helicase (WRN) protein and anti-WRN antibody

Cloning and purification of WRN protein and the preparation of anti-WRN antibody were described previously.[14,15] The purity of the recombinant WRN protein was ~80% determined by CBB staining.

Immunoblotting for the detection of anti-WRN antibody

Immunoblot analysis for autoantibody against RecQ3 RNA/DNA helicase was performed as follows. Recombinant RecQ3RNA/DNA helicase was electrophoresed on 2%–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and electroblotted to a nitrocellulose sheet. After blocking with bovine serum albumin/Tris–HCl buffer, 1:50 diluted SSc serum was incubated with the nitrocellulose sheet. After washing, 1:1000 diluted alkaline phosphatase-conjugated antihuman IgG antibody was added and incubated. The sheet was washed with the buffer, incubated with the substrate solution, and then washed thoroughly with deionized water.

Enzyme-linked immunosorbent assay (ELISA) for antibody binding to RecQ3 RNA/DNA helicase

Polystyrene microtiter plates were coated with 100 µl WRN protein (10 µl/ml in Tris–HCl buffer, pH 7.6) by overnight incubation at 4°C. After blocking with 300 µl 1% bovine serum albumin (BSA) in Tris–HCl buffer for 30 min, sera were diluted 1:100 and incubated overnight at 4°C in duplicate WRN-coated wells and duplicate BSA-coated wells. Bound antibody was detected with horseradish peroxidase (HRP)-conjugated goat anti-human IgG or IgM antibody (1:2000). The peroxidase substrate *ortho*-phenylenediamine and H_2O_2 was used as chromagen. Absorbance at 490 nm (A_{490}) was measured using an automated spectrophotometer as described previously.[16] The binding activities of the antibody are expressed as an index calculated by the following formula: Index = [sample (A_{490})–negative control (A_{490})] × 100/[positive control (A_{490})–negative control (A_{490})]. The serum with the highest anti-WRN antibody level was arbitrarily set as positive control (Index = 200).

Inhibition assay

The capacity of WRN protein to block antibody binding in the representative sera to solid-phase WRN protein was measured by adding increasing amounts of WRN protein (~40 µg/ml) to the sera with elevated IgG anti-WRN activity. The final serum concentration was 1:100, and the

incubation time at room temperature was 60 min. The residual IgG antibody-binding capacity to solid-bound WRN protein was determined by ELISA as described above.

ELISA for anti-topoisomerase I antibody

Serum IgG autoantibody against topoisomerase I (Scl-70) was measured by using a commercially available ELISA kit (MESACUP-2 test Scl-70; MBL, Nagoya, Japan). The data were expressed according to the manufacturer's suggestion.

Statistical analysis

Data are given as the mean and 2 standard deviations (mean±2 SD) or the median and interquartile range. Data analyses were performed using a statistical analysis software package (StatFlex; ViewFlex, Tokyo, Japan) to evaluate the Mann–Whitney *U*-test and Pearson's correlation test. Differences with *P* values less than 0.05 were considered to be statistically significant.

Results

Serum levels of anti-RecQ3 RNA/DNA helicase (WRN) in SSc

The median and interquartile (Q1–Q3) serum level of IgG anti-WRN antibody in the patients with SSc (131.2 and 89.1–170.0 Index, $n = 41$) was significantly higher than that in age- and sex-matched normal volunteers (71.2 and 58.0–105.5 Index, $n = 30$, $P < 0.001$, Wilcoxon *t*-test, Fig. 1a). The median and interquartile serum level of IgM anti-WRN antibody in the patients with SSc (79.4 and 51.1–111.7 Index, $n = 41$) was also significantly higher than that in age- and sex-matched normal volunteers (55.7 and 38.3–71.3 Index, $n = 30$, $P < 0.05$, Fig. 1b). Moreover, the median and interquartile serum level of IgG anti-WRN antibody in the patients with SSc of diffuse type (142.9 and 118.1–184.1 Index, $n = 30$) was significantly higher than that of limited type (92.7 and 80.1–97.2 Index, $n = 11$, $P < 0.01$, Fig. 2). Cutoff value for the positivity of IgG anti-WRN was arbitrarily settled based on the mean±2 SD (56.2 ± 30.6 = 86.8 Index) from normal volunteers ages between 20 and 60. Positive serum for IgG anti-WRN was 80% in diffuse type SSc, while less than 10% serum was IgG anti-WRN positive in limited type SSc and normal volunteers. Neither a significant correlation between IgG, IgM, or IgA anti-WRN antibody in the SSc sera with disease duration, patients' age, sex, clinical phenotypes other than scleroderma, nor type of therapy was observed (data not shown).

Relationship between serum levels of IgG anti-WRN and IgG anti-topoisomerase I antibody

A significant correlation was observed between serum levels of igG anti-WRN and anti-topoismerase I antibodies in the patients with SSc ($r = 0.363$, $n = 41$, $P < 0.05$; Fig. 3).

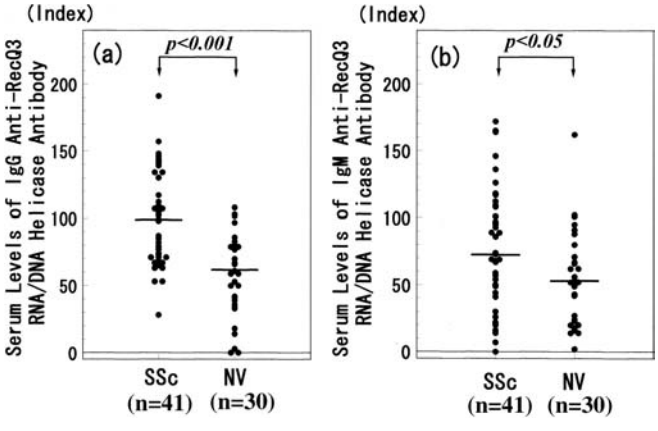

Fig. 1a,b. Anti-RecQ3 RNA/DNA helicase antibodies (anti-WRN) in systemic sclerosis (*SSc*). Serum levels of IgG (**a**) and IgM (**b**) anti-WRN in the patients with SSc ($n = 41$) were compared with those from age- and sex-matched normal volunteers (*NV*; $n = 30$). **a** The median (indicated by *bars*) serum level of IgG anti-WRN in SSc was 131.2 Index, while that of normal volunteers was 71.2 Index ($P < 0.001$). **b** The median serum levels of IgM anti-WRN in SSc were 79.4 Index and 55.7 Index in normal volunteers. The difference was also significant ($P < 0.05$)

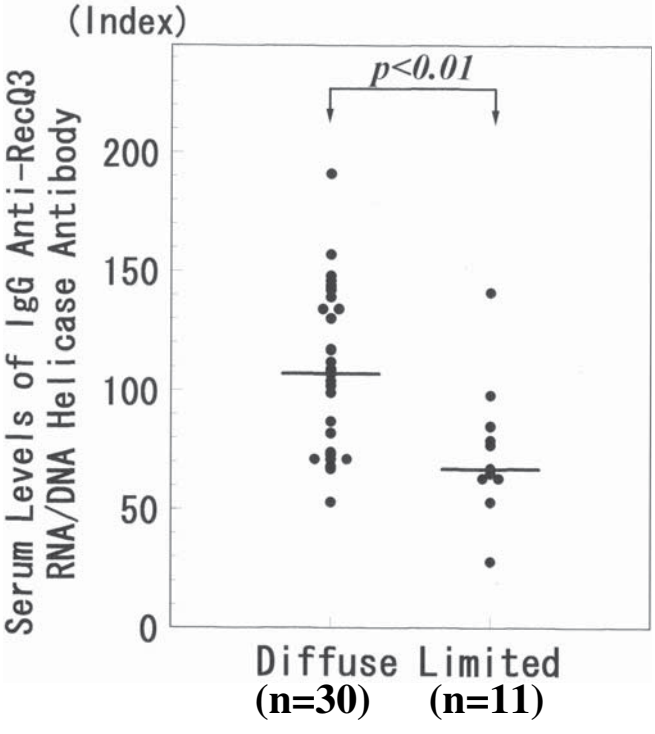

Fig. 2. Serum level of IgG anti-WRN in SSc of diffuse type and limited type. The median serum level of IgG anti-WRN in diffuse type SSc ($n = 30$) was 142.9 Index, while that of limited type SSc ($n = 11$) was 92.7. The difference was statistically significant ($P < 0.01$)

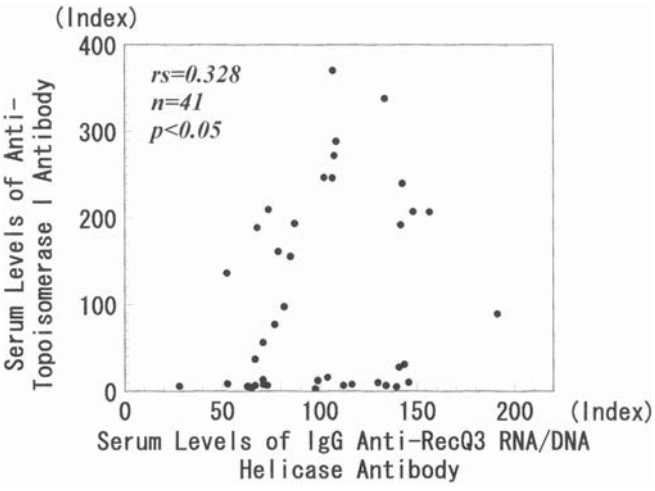

Fig. 3. Relationship between serum levels of IgG anti-WRN and anti-topoisomerase I antibody in patients with SSc. If the relationship between the IgG levels of anti-WRN and anti-topoisomerase I antibodies was examined in the same sera from SSc, a statistically significant correlation ($P < 0.05$) was observed

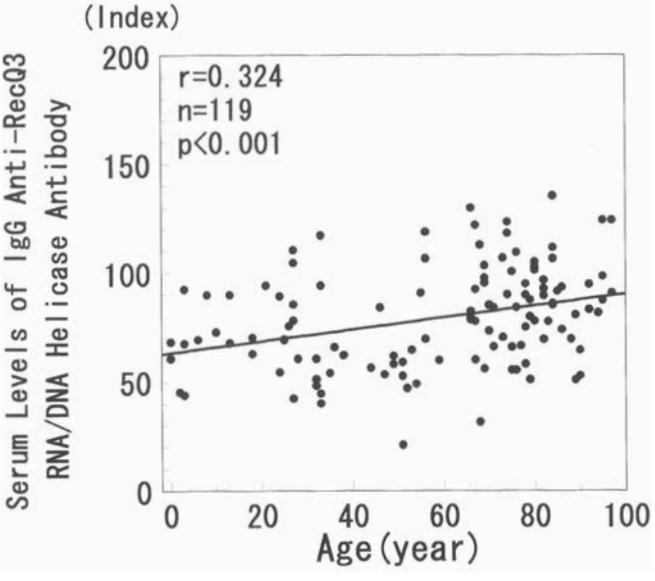

Fig. 4. Correlation between age and serum level of IgG anti-WRN in normal individuals. Age dependency of the serum IgG anti-WRN level among healthy individuals from 0 to 99 years old was significant ($P < 0.001$)

Serum level of IgG anti-WRN changes with age in the healthy population

In the 119 normal healthy individuals aged from 0 to 99 years, serum level of anti-WRN of IgG type correlated significantly with advancing age ($P < 0.001$) as depicted in Fig. 4. Serial analysis of IgG anti-WRN antibody in the same individuals for several years showed relatively constant levels (data not shown).

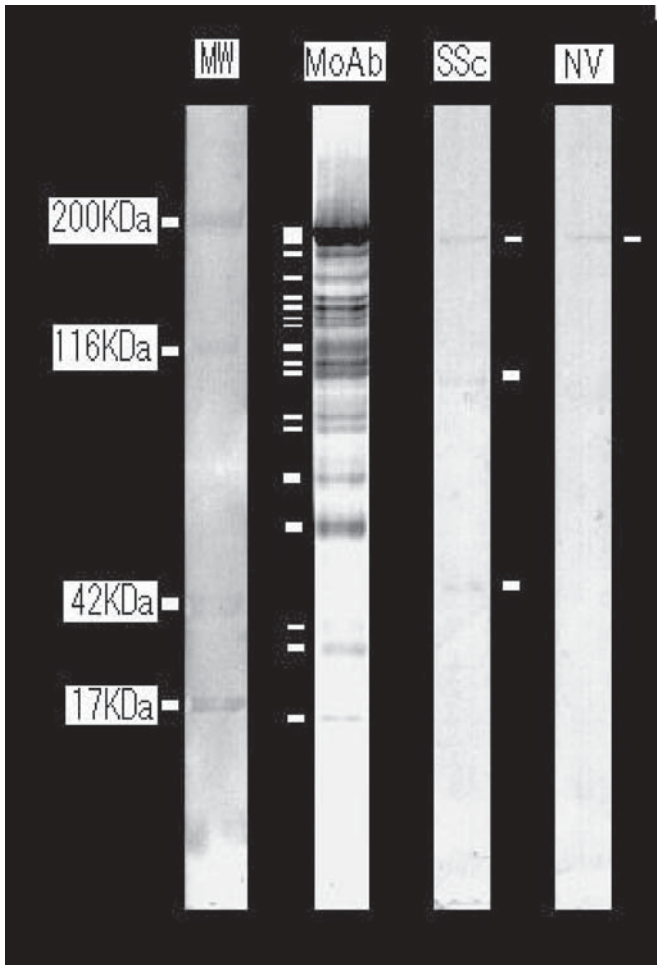

Fig. 5. Immunoblot analysis for reactivity of SSc serum with recombinant RecQ3 RNA/DNA helicase. Western blotting experiment showed a 200-kDa band specific to RecQ3 RNA/DNA helicase. *MW*, molecular weight marker; *MoAb*, monoclonal antibody against recombinant RecQ3 RNA/DNA helicase; *SSc*, systemic sclerosis; *NV*, normal volunteers

Specificity of the antibody to RecQ3 RNA/DNA helicase (WRN)

Western blotting showed a 200-kDa specific band of anti-WRN autoantibody from both SSc patients and normal volunteers to the recombinant WRN protein (Fig. 5). Inhibition of the specific binding capacity of anti-WRN antibody in the representative sera from both SSc patients and normal volunteers was observed by adding increasing amounts of recombinant WRN protein in a similar fashion, as shown in Fig. 6.

Discussion

Both SSc and WS have similar skin changes, i.e., scleroderma, hyper-/hypopigmentation, telangiectasia, skin ulcers, painful corns, and subcutaneous calcification, in addition to generalized fibrosis, though SSc has been believed

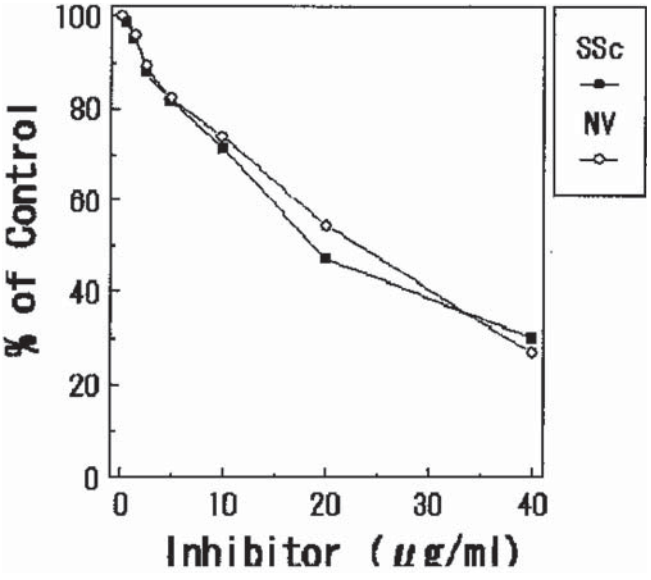

Fig. 6. Inhibition of IgG anti-WRN (RecQ3 RNA/DNA helicase antibody by RecQ3 RNA/DNA helicase protein. The sera from the representative SSc patient and a normal volunteer were incubated with an increasing amount (0–40 μg/ml) of recombinant RecQ3 RNA/DNA helicase protein at room temperature for 60 min. The residual IgG anti-RecQ3 RNA/DNA helicase antibody level was determined by the specific enzyme-linked immunosorbent assay. Inhibition of the specific binding of IgG anti-RecQ3 RNA/DNA helicase (WRN) of the SSc patients and normal volunteers to solid-phase coated recombinant RecQ3 RNA/DNA helicase protein was expressed as percent of control. Similar results were obtained using other SSc samples

to be the result of unidentified autoimmune mechanisms in contrast to the autosomal-recessively inherited WS caused by the loss of function of the mutated RecQ3 RNA/DNA helicase (WRN). We have been exploring common mechanisms in terms of connective tissue metabolism underlying between WS and SSs.[1] We have demonstrated for the first time that IgG autoantibody to a nucleolar RecQ3 RNA/DNA helicase (WRN) protein occurs in 80% of patients with diffuse type SSc, and may be associated with systemic skin sclerosis in SSc (Figs. 1 and 2). The presence of anti-WRN antibodies, however, is not specific for SSc, since positive serum was found in a separate experiment from other autoimmune diseases including systemic lupus erythematosus, rheumatoid arthritis, Sjögren syndrome, mixed connective tissue disease, and even aged normal individuals, though both the frequency and the titer are relatively low.

Systemic sclerosis is a relatively uncommon human disease characterized by progressive fibrosis of the skin and internal organs, including the lungs and gastrointestinal tract, with unknown etiology. Most scientists believe an inciting immunologic imbalance to be the primary cause of the disease, like other autoimmune diseases, but despite much research over the past half century, the underlying mechanisms remain unclear. One important clue to its etiology is the nearly universal association of disease with autoantibodies, which are antibodies directed against ubiquitous host proteins (autoantigens) such as topoisomearase I. RecQ3 RNA/DNA helicase is also a ubiquitous protein and functions in accordance with other enzymes such as topoisomearase I, II, and III, Ku, proliferating cell nuclear antigen, RNA polymerase I, II, and III in RNA/DNA metabolism.[8,17] The autoantibodies against these RNA/DNA enzymes are frequently detected in autoimmune diseases including SSc, though we still do not know whether their autoantibodies detected in the circulation are merely the byproduct of normal apoptosis or diseased damage and have something to do with etiology, or have any pathogenetic role in the autoimmune diseases. Some autoantibody has been shown to have a nuclear localization signal and may intrude into the nucleus, suggesting a possible coordination with RNA/DNA enzymes within the nucleus or nucleolus,[18,19] though we do not know if the IgG anti-WRN antibody has the nuclear localization signal or has a binding capacity to the cell membrane of fibroblasts in SSc like anti-topoisomearase I antibody, as reported previously.[20]

The role of IgG anti-WRN autoantibody in the pathogenesis of skin sclerosis is not clear. Elevated serum IgG is frequently detected in patients with SSc and aged individuals and may share common mechanisms with the production of IgG anti-WRN autoantibody. We would like to speculate a role of IgG anti-WRN in the skin atrophy followed by skin sclerosis tightly associated with the normal aging process and SSc.

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