ORIGINAL REPORT

Autoantibody Profile in Systemic Sclerosis

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Abstract- Systemic sclerosis is a generalized disorder of connective tissue clinically characterized by thickening and fibrosis of the skin and by distinctive forms of involvement of internal organs. One of the hallmarks of systemic sclerosis is the presence of serum autoantibodies against a variety of nuclear and cytoplasmic antigens. The primary purpose of this study was to identify the autoantibodies profile in the scleroderma sera and the secondary goal was to determine the correlation and discrepancy of autoantibody profile. Autoantibody profile was determined in 118 samples stored in the Advanced Diagnostic Laboratory at the University of Calgary. 78 sera were provided from Canadian and 40 sera were provided from Ukraine. We used the following techniques to identify autoantibodies profile in scleroderma patients: 1. Antinuclear antibody (ANA) by indirect immunofluorescence on human epithelial cell substrate 2. Detection and identification of specific autoantibodies by Innolia strip assay 3. Detection and identification of specific autoantibodies against extractable nuclear antigens. 111 out of 118 patients showed positive ANA results by indirect immunofluorescence and 7 patients had negative ANA results. Anti-ENA analyses by Inolia were positive in 84 patients, while by western blotting 81 patients showed positive results. In this study, we compared the results of anti-ENA antibody by Innolia with SLR technique. A significant correlation was found between anti-SCl-70 antibodies (P=0.000) and anti- RNP antibodies (P=0.001) and JO-1 antibodies (P=0.014). Thus, we may propose that SLR and Innolia techniques could be used for the detection of autoantibody in systemic sclerosis.

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Key words: Autoantibodies; scleroderma, systemic; nuclear antigens

Introduction

Systemic sclerosis is a generalized disorder of connective tissue clinically characterized by thickening and fibrosis of the skin and by distinctive forms of involvement of internal organs, notably the heart, lungs, kidneys and gastrointestinal tract (1). The etiology of systemic sclerosis is almost certainly multifactorial involving genetic and environmental factors (2). The incidence of systemic sclerosis is between 18-20 individuals per million of population per year (3, 4), the prevalence ranging from 0.1 to 13.8 per 100,000 (5-10). Systemic sclerosis is found in all geographic areas and all racial groups, although blacks may be moderately at an increased risk. It is three to four times more common in women than in men, with women of childbearing age being at the highest risk. The majority of cases occur sporadically without reference to season, geography, occupation or socioeconomic status (1). One of the hallmarks of systemic sclerosis is the presence of serum autoantibodies against a variety of nuclear and cytoplasmic antigens. Aoutoantibodies have been used extensively as a useful biomarker in autoimmune rheumatic diseases such as systemic sclerosis. Detection of antinuclear antibody by immunofluoresence is a standard clinical test to screen for evidence of systemic autoimmunity. Different specific atuoantibodies are associated with particular diagnosis, symptoms, unique syndromes, subsets of the disease and clinical activity. They are produced prior to the onset of clinical manifestation and are of predictive clinical value (11). Antibody profile has never been investigated in a large series of patients. The primary purpose of this study is to identify the autoantibody profile in the scleroderma sera and a secondary goal is to determine the correlation and discrepancy of autoantibody profile determined by three techniques: indirect immunofluores-

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cence, addressable laser bead immunoassay and a LINE assay.

Patients and Methods

Autoantibody profile was determined in 118 samples stored in the Advanced Diagnostic Laboratory at the University of Calgary, 78 sera were provided from Canadian patients.

Scleroderma study group and 40 sera were provided from Ukraine. We used the following techniques:

1. Antinuclear antibody (ANA) by indirect immunofluorescence (IIF) on human epithelial cell (HEP-2000) substrate (Immuno Concept, Sacramento, CA). Sera were diluted with PBS and the dilutions used for the ANA′s were 1/160 and 1/640. We added 24 µl sera of patients or controls to each well, then covered the chamber with the lid and incubated the slides for 30 minutes at room temperature. Each slide was washed quickly with 2 changes of PBS. The slides were then placed back in the humidifier chamber and over layered with diluted goat anti-human IgG fluorescein (FITC) conjugated antibody and incubated for 30 minutes. The slides were then washed in PBS three times for 5 minutes each, quickly and gently blotted slides overlaid with a coverslip and placed on the slide tray. The slides were stored at 4°C until being read on the Zeiss fluorescence microscope.
2. Detection and identification of specific autoantibodies by Innolia strip assay (Innogentics, Norcross, GA). Briefly, sera were diluted 1/200 in a sample diluent and then incubated for 1 hour with LIA test strips in a plastic holder. After 3 washes with wash solution, the strips were incubated with conjugate solution for 30 minutes. After washing with wash solution was add to sera and incubated for 30 minutes. The liquid was aspirated and 2 ml of stop solution was added to each trough and incubated for 20 minutes. After that, the strips were removed from the troughs and fixed on a data reporting sheet.

3. Detection and identification of specific autoantibodies to extractable nuclear antigens (ENA) with MARDX HEp-2 marblot system (SLR Research, Carlsbad, CA). This test is a western blot technique utilizing antigens extracted from Human HEp-2 cells. The marblot strips were removed from the strip vial with blunt forceps and placed in channels of a strip-incubated tray. A channel was filled with 2.0 ml sample diluent / wash solution and incubated for 5 minutes on a platform rocker. Then, 80 µl of each sera sample was added to the appropriate channel and incubated for 30 minutes. After decanting the contents, 2.0 ml of the diluted alkaline phosphotase conjugated anti-human IgG (MARDX) was pipeted into each channel and incubated by rocking for 15 minutes. After decanting the contents, 2.0 ml of sample diluent/ wash solution was added to each channel and incubated for 5 minutes in two changes of wash solution. Then 2.0 ml of deionized water, 2.0 ml of Alkaline phosphotase developing solution was added to each channel and incubated for 4-12 minutes or until the positive control had sufficiently developed color. The contents were decanted and 2.0 ml of deionized water was added to each channel of the incubation tray. The strips were removed from the channel with blunt forceps and care was taken not to expose the membrane to direct lighting for extended periods.

Statistical analysis:

Data was analyzed by SPSS software (version 11.5 for windows). The results were depicted in proper tables and charts. Contingency coefficient test was used for evaluating concordance of different laboratory tests. P-value<0.05 was considered significant.

Results

In the present study, we determined the autoantibody profile by three different techniques. Our results showed that 111 patients out of 118 (94%) were ANA positive and the remainder 7 (5.93%) were ANA negative. The predominant patterns on HEp-2 cell substrate were speckled (70%), nucleolar (37%), and homogenous (19.4%; Table 1).

We used Innolia and SLR methods for evaluating anti-ENA in the patients. SLR method was positive in 81 patients (68.6%) and negative in 37 (31.3%). Innolia method was positive in 84 and negative in 34 patients.

The prevalence of anti-SCL-70 in western blotting technique was 32% and by Innolia test was 29.6%. Anti-SCL-70 antibody occurred most frequently in our patients (Table 4, 5). The prevalence of anit-RNP autoantibody by western blotting technique was 25% and by Innolia test was 10% for RNP70 and 8.4% for RNP-A and 6.7% for RNP-C.

The prevalence of anti Sm autoantibody in western blotting technique was 21% and by Innolia test was 15%.

The prevalence of anti SSA52 and SSA60 autoantibody in western blotting technique were 16.9% and 16% and by Innolia test, these values were 18.6% and 9.3%, respectively.

Table 1. ANA result

# (%)	ANA Result
7 (5.93%)	Negative IIF
111 (94.06%)	Positive IIF
83 (70%)	Speckled pattern
44 (37%)	Nucleolar pattern
23 (19.4%)	Homogenous pattern
19 (16%)	Centromere pattern
9 (7.6%)	SSA /Ro pattern
9 (7.6%)	NSP (nuclear speckled) pattern
5 (4.2%)	AMA pattern (anti mitochondrial antibody)
1 (0.8%)	Ribosomal Antibody pattern
1 (0.8%)	DCS (Discrete Cytoplasm sp)

Table 2. ANA Result from Canadian patients

ID Patient	Pattern and Dilution
1	nucleolar 1/2560, speckled 1/1280
2	homogeneous & speckled 1/1280, nucleolar (speckled-type), 1/640
3	NSpI 1/1280, speckled 1/640
4	SSA/Ro positive by ANA, centromere 1/1280
5	NSpI 1/2560, speckled 1/1280
6	centromere 1/640, speckled 1/1280
7	SSA/Ro positive by ANA, speckled 1/160
8	ANA negative
9	speckled 1/640
10	nucleolar 1/160
11	centromere 1/640
12	ANA negative
13	speckled 1/2560
14	speckled 1/640, nucleolar (speckled-type), homogeneous 1/160
15	speckled 1/1280
16	homogeneous & speckled 1/2560, nucleolar (speckled-type), 1/640
17	centromere 1/1280, nucleolar 1/1280
18	nucleolar 1/2560, speckled 1/1280
19	homogeneous & speckled 1/1280, nucleolar (speckled-type), 1/640
20	NSpI 1/1280, speckled 1/640
21	homogeneous & speckled 1/640, nucleolar (speckled-type) 1/640
22	homogeneous & speckled 1/1280, nucleolar (speckled-type), 1/640
23	NSpI 1/640, speckled 1/160
24	centromere 1/2560, nucleolar 1/160
25	homogeneous & speckled 1/640, nucleolar)speckled-type (1/320
26	needs to be repeated
27	ANA negative
28	speckled 1/2560
29	homogeneous & speckled 1/160
30	homogeneous & speckled 1/1280, nucleolar (speckled-type) 1/640
31	speckled 1/160

32	speckled 1/640
33	speckled 1/640, nucleolar (speckled-type) 1/320
34	ANA negative
35	SSA/Ro positive by ANA, speckled 1/640, nucleolar 1/640
36	speckled 1/1280, nucleolar 1/640 (PM/Scl or speckled-type?)
37	speckled 1/160
38	centromere 1/1280
39	homogeneous & speckled 1/2560
40	homogeneous 1/2560
41	speckled 1/160, midbody 1/320
42	speckled 1/160, nucleolar (speckled-type) 1/320, SSA/Ro positive by ANA
43	homogeneous & speckled 1/1280, centromere 1/1280
44	centromere 1/1280
45	SSA/Ro positive by ANA, centromere 1/1280, speckled 1/160
46	nucleolar (speckled-type) 1/640, speckled 1/320, homogeneous 1/160
47	nucleolar 1/2560, speckled 1/640
48	homogeneous & speckled 1/1280, nucleolar (speckled-type), 1/640
49	nucleolar 1/640
50	nucleolar 1/160
51	centromere 1/2560
52	homogeneous & speckled 1/320, nucleolar (speckled-type) 1/160
53	nucleolar)speckled-type (1/640, speckled 1/160
54	NSpI 1/1280, speckled 1/1280
55	centromere 1/2560, speckled 1/1280
56	mitochondria 1/2560
57	NSpI 1/640, homogeneous & speckled 1/1280, ribosome 1/1280
58	speckled 1/640
59	SSA/Ro by ANA, centromere 1/2560, speckled 1/160
60	homogeneous & speckled 1/1280, nucleolar (speckled-type), 1/640
61	NSpI 1/1280, speckled 1/640
62	SSA/Ro positive by ANA, speckled 1/1280, nucleolar 1/320
63	homogeneous & speckled 1/2560, nucleolar (speckled-type), 1/2560
64	centromere 1/2560, speckled 1/640
65	homogeneous & speckled 1/1280, nucleolar (speckled-type), 1/640
66	homogeneous & speckled 1/1280, nucleolar (speckled-type), 1/640
67	centromere 1/1280, nucleolar 1/160
68	nucleolar 1/1280
69	speckled 1/640
70	speckled 1/640
71	NSpI 1/1280, speckled 1/1280
72	speckled 1/160
73	speckled 1/2560
74	centromere 1/2560
75	centromere 1/2560, nucleolar 1/160
76	nucleolar 1/160
77	homogeneous & speckled 1/1280, nucleolar (speckled-type), 1/640, mitochondria 1/640
78	nucleolar 1/640

The prevalence of anti SSB autoantibody in western blotting technique was 16% and by Innolia test was 11%.

The prevalence of anti Jo-1 autoantibody in western blotting technique was 6.7% and by Innolia test was 1.6%.

Using Innolia method, 30.65% (36 patients) were positive for SCL-70 autoantibody. 36.1% of these patients were negative for SCL-70 using SLR method and the remaining 63.9% were positive. The Innolia method was negative for SCL-70 in 69.5% of patients (82 cases). By SLR method, 81.7% of these patients were also SCL-70 negative and the remaining 18.3% were positive. Contingency coefficient test showed significant diagnostic concordance for SLR and Innolia methods (P <0.05).

Table 3. ANA result from Ukraine patient

ID Patient	Dilution	ANA Result	Titer
1	1/160	Trace sp	80
2	1/160	1-2+MSA,1 +sp,1 +nucleolar	160
3	1/160	4 +clumpy nucleolar	1280
4	1/160	3 +sp,3 +nucleolar may be PMSCL	1280
5	1/160	2+Nsp1,2+AMA=sp 100	320
6	1/160	1+sp	160
7	1/160	1-2 +sp	160
8	1/160	3+sp,3 +Nucleolar	1280
9	1/160	3-4+ho/sp,2+AMA	1280
10	1/160	Trace sp	80
11	1/160	2+sp	320
12	1/160	2 +ho/sp,1+Nucleolar(atipycal cytoplasmic sp)	640
13	1/160	2 +cytoplacmic sp	320
14	1/160	3+DCS(discrete cytoplasm sp)	1280
15	1/160	Neg	0
16	1/160	2 +Nucleolar	1280
17	1/160	3+sp Nucleolar	640
18	1/160	Trace sp	80
19	1/160	4 +sp may be 3+SSA	1280
20	1/160	2 +sp, 2+Nucleolar	640
21	1/160	4 +ACA	640
22	1/160	2-3 +AMA	640
23	1/160	1 +sp	160
24	1/160	1 +sp,1 +Nucleolar	160
25	1/160	Neg	0
26	1/160	4 +ACA,1 +sp	640
27	1/160	2 +cytoplasmic filament	160
28	1/160	Trace sp& Nucleolar	80
29	1/160	2+cytoplasmic sp	640
30	1/160	4+ACA	640
31	1/160	Trace sp& Nucleolar	80
32	1/160	2 +sp,3 +Nucleolar	640
33	1/160	3 +sp	1280
34	1/160	Neg	0
35	1/160	1 +sp	160
36	1/160	1 +sp may be1 +Nucleolar	160
37	1/160	Neg	00
38	1/160	2-3 +sp,1 +ho, may be SSA	1280
39	1/160	Trace sp	80
40	1/160	2 +sp	640

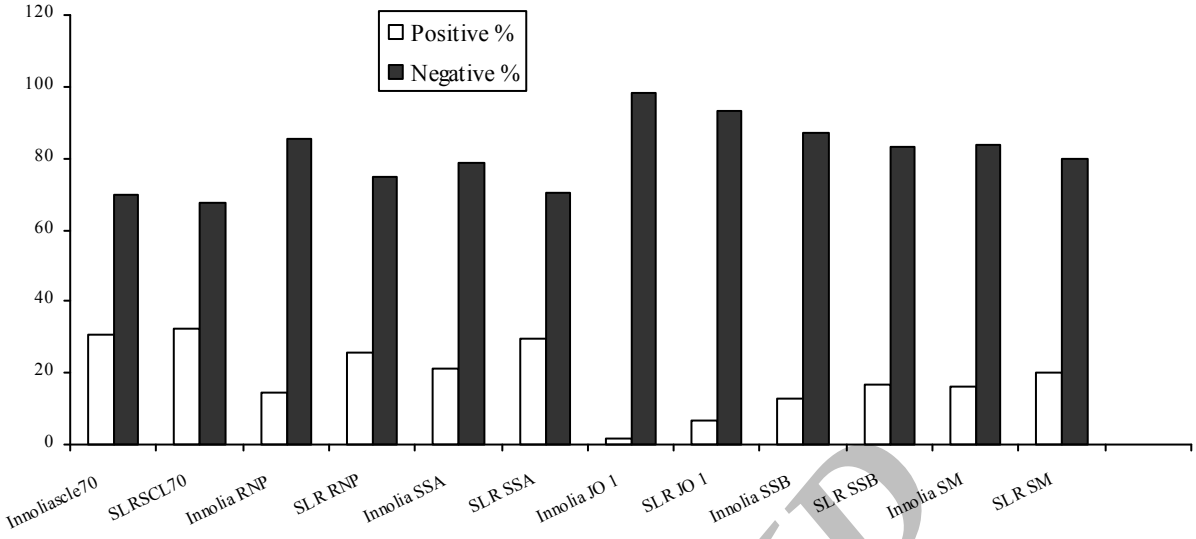

Figure 1. Autoantibody Frequency between SLR and Innolia Test

Using Innolia method, 14.4% (36 patients) were positive for RNP autoantibody. 41.2% of these patients were negative for RNP using SLR method and the remaining 58.8% were positive. The Innolia method was negative for RNP in 85.6% of patients (101 cases). By SLR method, 80.2% of these patients were also RNP negative and the remaining 19.8% were positive. Contingency coefficient test showed significant diagnostic concordance for SLR and Innolia methods (P <0.05).

Using Innolia method, 21.2% (25 patients) were positive for SSA autoantibody. 60% of these patients were negative for SSA using SLR method and the remaining 40% were positive. The Innolia method was negative for SSA in 78.8% of patients (93 cases). By SLR method, 73.1% of these patients were also SSA negative and the remaining 26.9% were positive. Contingency coefficient test didn't show significant diagnostic concordance for SLR and Innolia methods (P >0.05).

Using Innolia method, 1.7% (2 patients) were positive for Jo-1 autoantibody. 50% of these patients were negative for Jo-1 using SLR method and the remaining 50% were positive. The Innolia method was negative for Jo-1 in 98.3% of patients (116 cases). By SLR method, 94% of these patients were also Jo-1 negative and the remaining 6% were positive. Contingency coefficient test showed significant diagnostic concordance for SLR and Innolia methods (P <0.05).

Using Innolia method, 12.7% (15 patients) were positive for SSB autoantibody. 80% of these patients were negative for SSB using SLR method and the remaining 20% were positive. The Innolia method was negative for SSB in 87.3% of patients.

By SLR method, 83.5% of these patients were also SSB negative and the remaining 16.5% were positive. Contingency coefficient test did not show any significant diagnostic concordance for SLR and Innolia methods (P >0.05).

Using Innolia method, 16.1% (19 patients) were positive for Sm autoantibody. 63.2% of these patients were negative for Sm using SLR method and the remaining 36.8% were positive. The Innolia method was negative for Sm in 83.9% of patients (99 cases). By SLR method, 82.8% of these patients were also Sm negative and the remaining 17.2% were positive. It seems that further studies on more cases are required (P =0.05).

Innolia and SLR methods showed statistically significant difference for SCL-70, RNP, and Jo-1 autoantibodies (P <0.05), while there was no significant difference between these two methods for SSA and SSB (P >0.05).

Table 4. SLR test result

# (%)	Autoantibody Results
37 (31.3%)	Negative SLR
81 (68.6)%	Positive SLR
38 (32%)	Scl-70 autoantibody
30 (25%)	RNP autoantibody
25 (21%)	Sm autoantibody
20 (16.9%)	SSA52 autoantibody
19 (16%)	SSA60 autoantibody
19 (16%)	SSB autoantibody
8 (6.7%)	Jo-1 autoantibody

Table 5. Innolia test result

# (%)	Autoantibody Results
34 (28%)	Negative Innolia result
84 (71%)	Positive Innolia result
35 (29.6%)	Scl-70 autoantibody
22 (18.6%)	SSA52 autoantibody
18 (15%)	SmB autoantibody
17 (14.4 %)	Histone autoantibody
13 (11%)	SSB autoantibody
12 (10%)	RNP70 autoantibody
11 (9.3%)	SSA60 autoantibody
10 (8.4%)	RNP-A autoantibody
8 (6.7%)	RNP-C autoantibody
2 (1.6%)	Jo-1 autoantibody
1 (0.8%)	SMD autoantibody
0	Ribo-P autoantibody

Discussion

Our aim was to investigate the autoantibody profile in patients with SSC and to describe different autoantibodies associated with systemic sclerosis disease.

Our result showed that 94.6% of patients were ANA positive and 5.93% were ANA negative. Previous studies have suggested the prevalence of ANA to be 68% by indirect immunofluoresence on Hep2 cells in 220 cases of systemic sclerosis in France (12).

Tobing and coworkers have suggested that ANA test by immunofluorescence (Hep-2) was positive in 60-90% (13). Hesselstrand et al. showed that ANA was positive in sera of 84% SSC patients by IIF techniques (14). Sharma et al. demonstrated that ANA was positive in 89.1% of patients with SSC (15). Our findings are concordant with the previous published data (16-18).

Analysis by IIF-HEP 2000 was useful for primary screening of patients with SSC for antinuclear antibodies. Several studies have investigated the diagnostic potential of various autoantibody tests in SSC. We used Innolia and SLR methods for evaluating anti-ENA in patients with SSC. The prevalence of positive results of ENA from both methods was almost the same.

Anti topoisomerase 1 antibody (ATA) was studied in 118 SSC patients using Innolia and SLR tests. Anti SCL-70 antibodies (ATA) are very useful in distinguishing systemic sclerosis patients from healthy controls, patients with other connective tissue disease, and un affected family members (19). Autoantibodies against topoisomerase (SCL-70) are important for the diagnosis of the disease and give clues for its clinical manifestation and prognosis (20). ATA can be identified through different techniques including double immunodiffusion assay, enzyme-linked immunosorbent assay or immune blot. Although all of them are commonly used, none can be considered as a reference highly specific for SSC (21). Similar to the previous studies, our result showed that about 30% of patients were positive for SCL-70 autoantibody (22) and there was good correlation between the two methods for SCL-70. Itulian study was designed to assess the analytical sensitivity and rate of agreement between ELISA and immune blot method in patients with systemic connective tissue disease. Overall agreement between the test reagents, for each anti ENA specificity, was 29% for RNP and for La/SSB.

SSA autoantibody results showed the same results as the previous studies (22-28). The only discordant finding compared to previous papers was Sm antibody using SLR method, which was positive in 20% of patients and in Innolia methods was positive in 16% patients while negative in similar studies in systemic sclerosis patients (29). This study described different autoantibodies associated with SSC disease. Despite the diagnosis of scleroderma is mainly clinical, these autoantibodies can constitute a diagnostic tool.

Identifying these autoantibodies requires a diagnostic strategy with two steps: the indirect immunofluorescence remains the best means of ANA tracking, with other specific identification methods, such SLR and Innolia, standing the second.

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