New Centromere Autoantigens Identified in Systemic Sclerosis Using Centromere Protein Microarrays

GUANG SONG, CHAOJUN HU, HENG ZHU, LI WANG, FENGCHUN ZHANG, YONGZHE LI, and LIN WU

ABSTRACT. ***Objective.*** To identify novel centromere protein (CENP) targets of anticentromere antibodies (ACA), and to investigate their association with clinical manifestations of systemic sclerosis (SSc).

Methods. A CENP-focused protein microarray was fabricated by spotting 14 purified CENP. These microarrays were individually incubated with 35 ACA-positive SSc sera and 20 ACA-negative healthy control samples. Newly identified CENP autoantigens with high sensitivities were selected for validation and characterization.

Results. Statistical analysis revealed 11 CENP are potential target antigens of ACA in patients with SSc. Of them, 5 [CENP-P, CENP-Q, CENP-M (isoform I), CENP-J, and CENP-T] are novel, among which CENP-P and CENP-Q showed high sensitivities in ACA-positive SSc sera of 34.3% and 28.6%, respectively. Subsequently, 186 SSc sera (35 ACA-positives and 151 negatives), 69 ACA-positive sera from other various autoimmune diseases (primary Sjögren syndrome, systemic lupus erythematosus, rheumatoid arthritis, and primary biliary cirrhosis), and 31 healthy sera were assayed for the presence of anti-CENP-P and -Q autoantibodies by ELISA followed by Western blotting analysis. CENP-P and -Q autoantibodies were detected in ACA-positive sera of various disease groups; among them, SSc showed the highest detection rate. Anti-CENP-P was also found in 9 of the 151 ACA-negative sera. Analyses of the correlation with clinical information showed anti-CENP-P-positive patients had higher levels of IgG, IgA, and erythrocyte sedimentation rate among the ACA-positive cohort and were more vulnerable to renal disease in the ACA-negative patients with SSc. Regardless of ACA status, anti-CENP-P or Q-negative patients seem to be predominantly affected by interstitial lung disease.

Conclusion. CENP-P and CENP-Q were identified as novel ACA autoantigens by CENP microarray assays followed by validation of ELISA and Western blotting. Both of them have prognostic utility for interstitial lung disease. CENP-P was associated with renal disease in an ACA-negative cohort. (J Rheumatol First Release Feb 15 2013; doi:10.3899/jrheum.120264)

Key Indexing Terms:
ANTICENTROMERE ANTIBODY
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Anticentromere antibodies (ACA) were first described in 1980 by incubation of human epithelial cell line 2 (HEp-2) cell substrates with the sera of patients with CREST syndrome (calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasias) using indirect immunofluorescence (IIF)[1]. The IIF pattern normally shows discrete speckled nucleoplasmic staining in HEp-2 cells at the interphase and distinct centromeric dots that are visible for each chromosome pair in the metaphase plate. ACA have been repeatedly demonstrated as useful biomarkers in the diagnosis of systemic sclerosis (SSc), where their sensitivity is about 20%–35%. ACA are closely associated with CREST syndrome, a limited form of SSc, and can also serve as predictors of a more benign and protracted course[2]. In addition, ACA are thought to be associated with pulmonary arterial hypertension[3,4] and cardiac conduction blocks[5].

Other than in SSc, ACA have been found in several other autoimmune diseases and cancers such as primary biliary cirrhosis (PBC; 18%–26.1%)[6,7,8], primary Sjögren syndrome (pSS; 3.7%)[9], systemic lupus erythematosus

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(SLE; 1.9%–11%)[10,11,12], rheumatoid arthritis (RA; 3%)[11], and breast cancer[13].

To date, several centromere proteins (CENP) have been identified as ACA autoantigens, among which CENP-B, CENP-A, and CENP-C are predominant. Immunostrips and/or ELISA assays using recombinant CENP-A and CENP-B have been used in some clinics for detection of ACA. Other known target antigens of ACA include CENP-D[14,15], CENP-E[16], and CENP-O[17] discovered in SSc, and CENP-F[18], CENP-G[19], CENP-H[20], and CENP-I[21] in other diseases. However, a recent study tested 95 sera with ACA, and found none reacted to CENP-H, -I, -K, -L, -M, -N, and –U, and only 1 reacted weakly to CENP-T[22]. Additionally, autoantibodies against several CENP have been associated with clinical manifestations, for example, the level of anti-CENP-B antibodies correlated with a less extensive skin involvement in SSc[23], and pSS patients with anti-CENP-H antibodies had a lower frequency of rheumatoid factor than those without[20]. Additionally, Gelber, *et al* found that the dual presence of anti-CENP-B and -C was most frequently seen in SSc, while anti-CENP-C alone was predominantly found in patients with pSS, and further suggested that obtaining antibodies to specific centromere antigens was diagnostically useful[24].

During eukaryotic cell division at the centromere locus, a multiprotein complex known as the kinetochore is assembled, which involves many CENP such as -A, -C, -H, -M, -N, -T, and MLF1IP/CENP-U in the CENP-A-NAC complex, and -I, -K, -L, -O, -P, -Q, -R, and -S in the CENP-A-CAD complex that interacts with the CENP-A-NAC complex[25]. However, whether other CENP have autoimmune activity has not been comprehensively surveyed.

To address this question, we first used a CENP-focused protein microarray composed of 14 CENP (A, B, C, H, I, J, K, L, M, N, O, P, Q, T) to profile ACA-positive SSc sera judged by IIF and/or immunostrip as in clinical practice. The new candidate CENP autoantigens identified by the microarrays were further tested by ELISA and Western blotting (WB) to validate the findings and to analyze their association with clinical manifestations.

MATERIALS AND METHODS

Serum samples. A total of 186 Chinese patients (171 women, age 44.6 ± 12.3 yrs) with SSc were enrolled. All of them fulfilled the American College of Rheumatology (ACR) classification criteria for SSc[26], and were subcategorized into limited SSc and diffuse SSc according to the classification system proposed by LeRoy, *et al*[27]. Also included were sera from 31 healthy volunteers (12 women, age 38.3 ± 11.9 yrs) and 69 ACA-positive patients affected by various autoimmune diseases including 18 pSS (all women, age 54.7 ± 10.2 yrs), 18 PBC (17 women, age 58.7 ± 11.4 yrs), 20 SLE (all women, age 44.7 ± 15.7 yrs), and 13 RA (11 women, age 59.4 ± 13.0 yrs). PBC was diagnosed according to the criteria from the American Association for the Study of Liver Diseases[28]; pSS fulfilled the American-European Consensus Group Classification criteria[29]; SLE and RA fulfilled the corresponding classification criteria from the ACR[30,31].

All samples were collected between January 2008 and December 2009 at Peking Union Medical College Hospital. Informed consent in writing was obtained from each participant. Our study was conducted with the approval of the Ethics Committee of the Peking Union Medical College Hospital.

Clinical measurements. Clinical assessment of organ manifestation in SSc was performed according to the report from the European League Against Rheumatism[32]. In short, the definitions of systemic involvement are as follows: cardiac involvement (arrhythmia and conductive block as revealed by electrocardiogram, systolic/diastolic dysfunction, pericardial effusion, and pulmonary arterial hypertension, estimated pulmonary arterial systolic pressure > 40 mm Hg, by echocardiogram); lung involvement [bilateral basilar velcro sounds by auscultation, evidence of interstitial lung disease (ILD) as demonstrated by chest radiograph, high-resolution computerized tomography (HRCT), and pulmonary function test (total lung capacity < 70% of predicted value, DLCO < 70% of predicted value)]; renal involvement (symptoms of nephrogenic peripheral edema, decreased daily urine output, and renal crisis diagnosed by clinician); and gastrointestinal manifestations (dysphagia, sour reflux, vomiting, diarrhea, constipation, and abdominal distension and decreased bowel sounds by auscultation).

Additionally, we analyzed the levels of IgG and IgA as well as erythrocyte sedimentation rate (ESR) for each patient with SSc. These are considered important indicators of autoimmune disease activity.

Detection of ACA. All samples were subjected to IIF and immunoblot testing using commercial kits according to the manufacturer's protocol (EuroImmun Medizinische Labordiagnostika AG). For IIF on HEp-2 cells, serum samples were considered ACA-positive at titers ≥ 160, and all negative sera showed no staining of ACA-specific pattern at titers of 1:40 and 1:80. The immunoblot strips contain CENP-A, CENP-B, and other SSc-related autoantigens including Scl-70, RNA polymerase, U3-RNP, and Th/To. The serum samples that were positive by IIF, CENP-A, or CENP-B were determined as ACA-positive. In total, 35 from the 186 SSc sera samples were identified as ACA-positive. Of them, 30 were positive by all 3 measures, 2 by both CENP-A and CENP-B, 1 by both CENP-B and IIF, and 1 each by CENP-B or IIF only.

Preparation of CENP and construction of CENP microarrays. A total of 16 recombinant proteins for 14 CENP (CENP-M and CENP-N have 2 isoforms each) were expressed and purified as glutathione S-transferase (GST) fusions in yeast as described[33]. All the purified CENP, together with controls (printing buffer, GST, nucleoprotein of influenza, and human IgG), were printed in triplicate within 12 identical probe areas on each OPEpoxySlide (CapitalBio Corp.). The CENP microarrays were stored at 4°C under vacuum until used.

Serum profiling with CENP microarrays. Thirty-five ACA-positive SSc sera and 20 ACA-negative healthy sera samples were individually incubated with the CENP microarrays, and the bound antibodies were detected following the procedure described[4]. We set the cutoff at 3 SD above the average signal intensity from healthy sera. The CENP were considered positive only when at least 2 of the triplicates were positive.

The 96-well plate was coated at 4°C overnight with recombinant CENP-P and CENP-Q proteins at 200 ng and 50 ng/100 □well, respectively. The ELISA was carried out as described[34]. The optical density (OD) at 450 nm was measured and the mean OD of the duplicate wells for each serum sample was used for data analysis. Because the CENP proteins are GST-fused, ELISA using equal molar amounts of GST protein were also conducted in parallel. The GST readings were subtracted from the readings of CENP. Cutoff values were set at 5 SD above the mean value from healthy samples.

Western blot analysis. Five hundred nanograms of recombinant GST-tagged CENP-P or -Q were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred onto PVDF membranes (Millipore). After being blocked with 5% nonfat milk, the membranes were incubated with sera (1:500), followed by horseradish peroxidase-conjugated anti-human IgG. The immunoreactive bands were visualized by chemiluminescence (Beijing Applygen Ltd. Co.).

Statistical analysis. P values were calculated with Pearson's chi-square or

Fisher's exact test using the R programming language and the Student t test if necessary. P values < 0.05 were considered statistically significant.

RESULTS

Construction of CENP protein microarray. Sixteen open reading frames (ORF) representing 14 CENP (A, B, C, H, I, J, K, L, M, N, O, P, Q, T; 2 ORF for both M and N) cloned into the pEGHA vector (supplementary data for 16 ORF available from the author upon request) were expressed and purified as GST fusions in yeast. Subsequently, each CENP, together with negative and positive controls (Figure 1A), was printed in triplicate within 12 identical blocks on a single slide to produce the CENP microarray. To evaluate the quality of the microarrays, an anti-GST monoclonal antibody was used to visualize and quantify the CENP immobilized on the slides (Figure 1B). All CENP on the microarray produced GST signals significantly above background and each feature showed highly reproducible signals with pairwise correlation coefficients of 0.999, indicating that this microarray is of high quality (Figure 1, B-C).

Identification of new CENP antigens for ACA by protein microarray. To determine whether additional CENP could be recognized by ACA, 35 ACA-positive SSc sera were individually incubated with the CENP microarrays (Figure 1, D-I). As a comparison, a cohort of 20 ACA-negative healthy subjects was also assayed in the same fashion. Using the mean value plus 3 SD of the healthy subjects as a cutoff, 11 of the 16 CENP showed various positive rates across the panel of ACA-positive SSc sera (Figure 2; supplementary data available from the author upon request), and none of the healthy sera were scored as positive. As expected, the 3 prominent antigens, CENP-B, CENP-A, and CENP-C, showed the highest positive rates of 97.1% (34/35), 77.1%

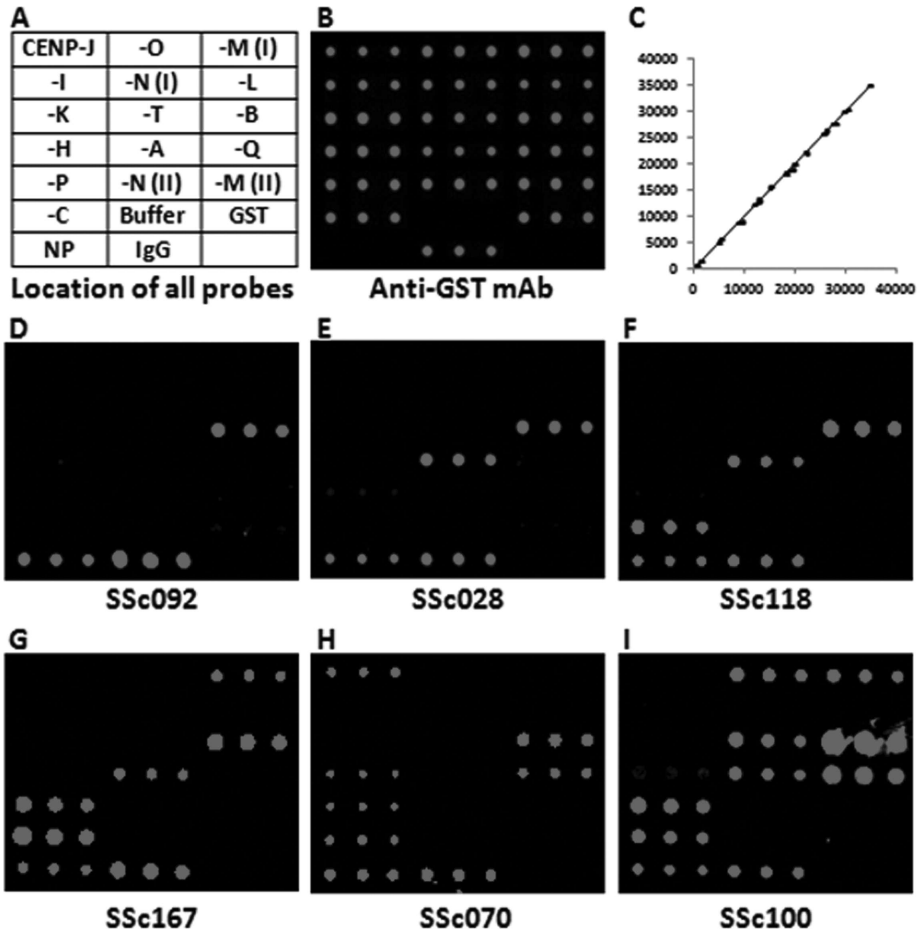

Figure 1. Profiling systemic sclerosis (SSc) sera with centromere protein (CENP) microarrays. A. Layout of CENP microarray. Each probe was printed in triplicate. M(I), M(II), and N(I), N(II) stand for isoform I and isoform II of CENP-M and CENP-N, respectively. NP (nucleoprotein of influenza), IgG, GST, and buffer are various controls. B. Scanned image of the CENP microarray probed with anti-GST antibody. The 2 negative controls (NP and buffer) did not show any signals, while all the other proteins, including IgG, showed strong and rather even signals. C. Reproducibility of triplicate protein probes detected by anti-GST antibody shown in B. The signal intensities of any 2 of the triplicate spots are plotted, and the correlation coefficiency is 0.9991, indicating good quality. D-I. Representative images of the CENP microarrays probed with anticentromere antibody-positive SSc sera. mAb: monoclonal antibody.

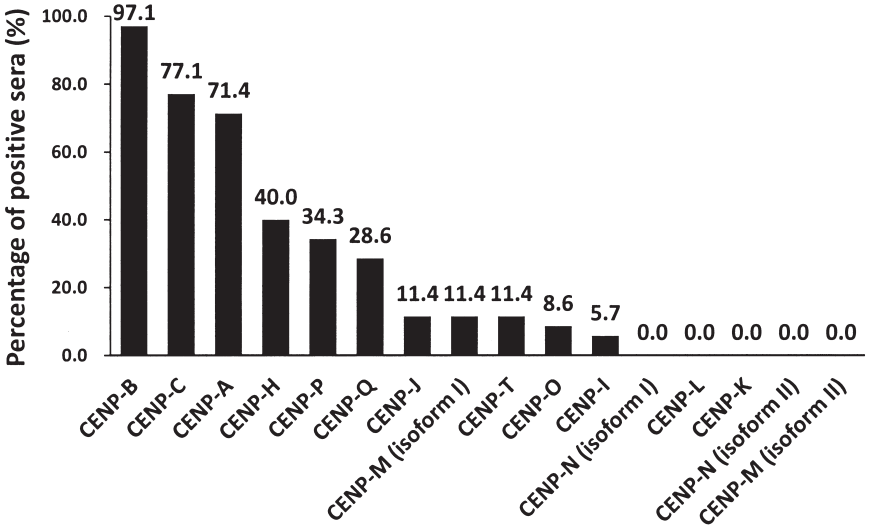

Figure 2. Percentage of anticentromere antibody (ACA)-positive systemic sclerosis (SSc) sera positive to each centromere protein (CENP) in the CENP microarray analysis. Thirty-five ACA-positive SSc sera samples were incubated with CENP microarrays, and the percentage of the positive sera for each CENP is plotted.

(27/35), and 71.4% (25/35), respectively, while 3 other known antigens, CENP-H, CENP-O, and CENP-I, could be recognized by 40% (14/35), 8.6% (3/35), and 5.7% (2/35) of the samples. Importantly, we were able to identify 5 new candidate ACA autoantigens [CENP-P, -Q, -J, -M (isoform I), and –T] that showed positive rates of 34.3% (12/35), 28.6% (10/35), 11.4% (4/35), 11.4% (4/35), and 11.4% (4/35), respectively.

Validation of CENP-P and CENP-Q autoantibodies by ELISA and Western blot. To validate the newly identified autoantigens, CENP-P and -Q were selected for further analysis because of their relatively high sensitivities. Sera from 186 patients with SSc (35 ACA-positive and 151 ACA-negative) and 31 healthy individuals (ACA-negative) were used to conduct the ELISA assay (supplementary data available from the author upon request). In total, 31 and 12 SSc sera were positive for CENP-P and -Q, respectively (supplementary data available from the author upon request). To validate them, all of these positives were subjected to WB using recombinant CENP-P and -Q proteins. We also included GST protein because CENP-P and -Q proteins were GST-tagged. No significant signals were observed for the GST protein (~26 kDa) for all samples (Figure 3). Twenty-three and 11 sera gave expected immunoreactive bands at 58–62 kDa for CENP-P and CENP-Q, respectively, and were considered true positives (Figure 3, Table 1).

For ACA-positive SSc sera, CENP-P and -Q showed a respective positive rate of 40% (14/35) and 31.4% (11/35), slightly higher than the microarray results. Interestingly, of the 151 ACA-negative SSc sera, 9 were CENP-P positive.

It is well known that ACA are not unique to SSc; many patients with other autoimmune diseases have them, although with lower frequency[6,7,8,9,10,11,12]. To comprehensively characterize the behavior of CENP-P and -Q autoantibodies in these autoimmune diseases, we also included ACA-positive sera from 18 patients with pSS, 18 with PBC, 20 with SLE, and 13 with RA (Figure 3, Table 1). Not surprisingly, CENP-P and -Q autoantibodies were detected in pSS, SLE, RA, and/or PBC at various rates.

Association of CENP-P and CENP-Q autoantibodies with clinical manifestations and laboratory features in SSc. To determine whether the newly identified autoantigens, CENP-P and -Q, have any diagnostic value for SSc, we performed additional statistical tests (chi-square/Fisher's exact test and T-test) to examine potential associations between the CENP-P or -Q autoantibodies and clinical manifestations and/or other laboratory features.

The analysis for anti-CENP-P is summarized in Table 2. For ACA-positive SSc sera, the serum levels of IgG and IgA in the patients with anti-CENP-P were significantly higher than those of patients negative for anti-CENP-P ($p < 0.05$). Meanwhile, ESR in patients who were anti-CENP-P-positive was also significantly higher than in patients who were anti-CENP-P-negative ($p = 0.0075$). For ACA-negative SSc sera, the percentage of patients with renal involvement was significantly higher in anti-CENP-P-positive patients than in anti-CENP-P-negative patients ($p = 0.042$). Additionally, regardless of ACA status, patients with SSc who were positive for anti-CENP-P showed significant association with renal involvement ($p = 0.0418$) but were less affected by ILD ($p = 0.0218$). As well, the mean age of these patients seems to be higher than that of patients without anti-CENP-P (mean age ± SD 49.9 ± 13.5 vs 43.8 ± 12 yrs; $p = 0.0267$). Further, anti-CENP-P was prevalent in both anti-CENP-A and –B-positive cohorts ($p < 0.0001$), although there were

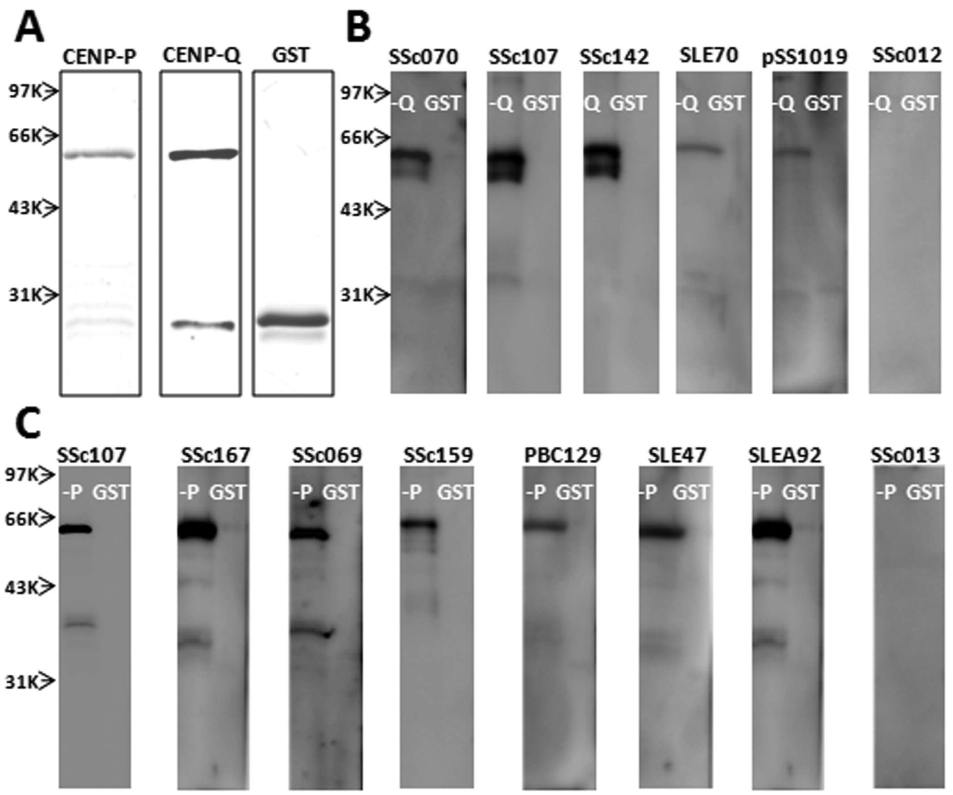

Figure 3. Validation of centromere protein (CENP)-P and CENP-Q autoantibodies by Western blotting. A. Recombinant GST-tagged CENP-P, CENP-Q, and GST proteins in SDS-PAGE. B. Blotted recombinant CENP-Q (58.6 kDa) and GST (28 kDa) proteins were probed with representative anti-CENP-Q ELISA-positive (SSc070, SSc107, SSc142, SLE70, and pSS1019) and -negative (SSc012) sera. C. Blotted recombinant CENP-P (61.2 kDa) and GST proteins were probed with representative anti-CENP-P ELISA-positive (SSc107, SSc167, SSc069, SSc159, PBC129, SLE47, and SLEA92) and -negative (SSc013) sera. GST: glutathione S-transferase; SSc: systemic sclerosis; SLE: systemic lupus erythematosus; PBC: primary biliary cirrhosis.

Table 1. Positive sera against centromere protein (CENP)-P and -Q in systemic sclerosis (SSc) and other cohorts. Positives to CENP-P/-Q were determined by ELISA and Western blot methods. P value was calculated using Pearson's chi-square or Fisher's exact test. P values < 0.05 are shown in bold type, indicating statistically significant differences compared to anticentromere antibody (ACA)-positive.

Cohort	Cases	Anti-CENP-P (+) No. (%, p value)	Anti-CENP-Q (+) No. (%, p value)
SSc			
ACA+	35	14 (40)	11 (31.4)
ACA–	151	9 (6, **< 0.0001**)	0 (0, **< 0.0001**)
pSS (ACA+)	18	1 (5.6, **0.0207**)	2 (11.1, 0.1771)
SLE (ACA+)	20	3 (15, 0.1038)	5 (25, 0.7608)
RA (ACA+)	13	2 (15.4, 0.1703)	0 (0, **0.0228**)
PBC (ACA+)	18	5 (27.8, 0.5644)	1 (5.6, **0.0412**)
Healthy (ACA–)	31	0 (0, **0.0002**)	0 (0, **0.0020**)

SLE: systemic lupus erythematosus; pSS: primary Sjögren syndrome; RA: rheumatoid arthritis; PBC: primary biliary cirrhosis.

still 39.1% and 43.5% of sera samples with anti-CENP-P that did not have anti-CENP-B and -A, respectively.

As for anti-CENP-Q (Table 3), its presence did not show significant association with any clinical manifestations and laboratory features in the ACA-positive cohort. However, regardless of ACA status, the presence of anti-CENP-Q was strongly associated with limited SSc and showed strong association with a lower prevalence of ILD (p = 0.003). In relation to other SSc autoantibodies, all the sera with anti-CENP-Q were also positive for both anti-CENP-B and -A, but anti-CENP-Q was more prevalent in the cohort negative for anti-Scl70 (p = 0.0244), an autoantibody associated with diffuse SSc.

DISCUSSION

Protein microarray technology has emerged as a high-throughput method for autoantigen identification, and has been successfully used for profiling autoantibodies in different types of diseases[34,35,36]. For patients with SSc, ACA have been shown to be useful diagnosis biomarkers for phenotypic subsets, as well as prognosis markers[37]. We fabricated a CENP-focused protein microarray to comprehensively survey their autoimmunity status in patients with SSc. In addition to the recovery of 6 known ACA antigens, 5 CENP were identified as potential novel autoantigens in SSc. Validation results from ELISA and Western blotting demonstrated that both CENP-P and -Q are actual centromere

Table 2. Association analysis between anti-CENP-P antibody in systemic sclerosis (SSc) sera and clinical manifestations and laboratory features. Bold type indicates p values < 0.05, considered statistically significant. Data are percentages unless indicated otherwise.

	ACA (+)			ACA (–)			All		
Anti-CENP-P	+	–	p*	+	–	p*	+	–	p*
No. Cases	14	21		9	142		23	163	
Disease pattern, limited/diffuse	12/2	17/4	1.0000	4/5	70/72	1.0000	16/7	87.76	0.2156
Sex, men/women	1/13	1/20	1.0000	0/9	13/129	1.0000	1/22	14/149	0.6983
Age, mean ± SD, yrs	50.9 ± 13.6	45.5 ± 7.8	0.1461	48.4 ± 14.0	43.6 ± 12.5	0.2653	49.9 ± 13.5	43.8 ± 12	**0.0267**
Anti-CENP-B	100	95.20	1.0000	0.0	0.0	1.0000	60.9	12.3	**< 0.0001**
Anti-CENP-A	92.90	90.50	1.0000	0.0	0.0	1.0000	56.5	11.7	**< 0.0001**
Anti-Scl-70	14.30	9.50	1.0000	44.4	52.1	0.7395	26.1	46.6	0.1025
Raynaud phenomenon	100	95.20	1.0000	100	94.4	1.0000	100.0	94.5	0.6042
Arthralgia/arthritis	35.70	33.30	1.0000	77.8	55.6	0.3010	52.2	52.8	0.8648
Digital ulcers	21.40	19	1.0000	22.2	29.6	1.0000	21.7	28.2	0.6872
Muscle weakness	21.40	19	1.0000	33.3	32.4	1.0000	26.1	30.7	0.8366
Gastrointestinal manifestations	14.30	4.80	0.5508	22.2	22.5	1.0000	17.4	20.2	1.0000
Cardiac involvement	7.10	9.50	1.0000	22.2	7.7	0.1745	13.0	8.0	0.4245
Renal crisis	7.10	0	0.4000	0.0	2.1	1.0000	4.3	1.8	0.4129
Kidney involvement	7.10	0	0.4000	22.2	2.8	**0.0420**	13.0	2.5	**0.0418**
Lung involvement	0	4.80	1.0000	44.4	21.1	0.1159	17.4	19.0	1.0000
Interstitial lung disease	28.60	33.30	1.0000	77.8	79.6	1.0000	47.8	73.6	**0.0218**
Pulmonary arterial hypertension	0	9.50	0.5059	33.3	17.6	0.3687	13.0	16.6	1.0000
IgG, mean ± SD g/l	16.7 ± 6	13.3 ± 2.9	**0.0463**	18.3 ± 5	16.7 ± 6.6	0.5238	17.3 ± 5.6	16.3 ± 6.3	0.5185
IgA, mean ± SD g/l	3 ± 1.3	2.2 ± 0.8	**0.0288**	2.4 ± 0.8	2.8 ± 1.3	0.4032	2.8 ± 1.2	2.7 ± 1.3	0.7055
IgM, mean ± SD g/l	1.8 ± 1	1.6 ± 1	0.7164	1.2 ± 0.3	1.4 ± 0.7	0.5531	1.6 ± 0.9	1.4 ± 0.7	0.3272
ESR, mean ± SD mm/h	25.9 ± 20.2	10.1 ± 9.2	**0.0075**	25.3 ± 15.3	24.4 ± 26.1	0.9238	25.7 ± 18.2	22.7 ± 25	0.5863

* P values calculated using Pearson's chi-square or Fisher's exact test and 2-tailed T-test if necessary. CENP: centromere protein; ESR: erythrocyte sedimentation rate; ACA: anticentromere antibodies.

Table 3. Association analysis between anti-CENP-Q antibody in systemic sclerosis sera samples and clinical manifestations and laboratory features. Bold type indicates p values < 0.05, which are statistically significant. Data are percentages unless otherwise indicated.

	ACA (+)			All		
Anti-CENP-Q	+	–	p*	+	–	p*
No. Cases	11	24		11	175	
Disease pattern, limited/diffuse	10/1	19/5	0.6400	10/1	93/82	**0.0243**
Sex, men/women	1/10	1/23	0.5361	1/10	14/161	1.0000
Age, mean ± SD, yrs	49.2 ± 12.7	46.9 ± 9.8	0.5674	49.2 ± 12.7	44.3 ± 12.3	0.2040
Anti-CENP-B	100	95.8	1.0000	100	13.1	**< 0.0001**
Anti-CENP-A	100	87.5	0.5361	100	12.0	**< 0.0001**
Anti-Scl-70	9.1	12.5	1.0000	9.1	46.3	**0.0244**
Raynaud phenomenon	90.9	100	0.3143	90.9	95.4	0.4295
Arthralgia/arthritis	36.4	33.3	1.0000	36.4	53.7	0.4198
Digital ulcers	18.2	20.8	1.0000	18.2	28.0	0.7300
Muscle weakness	36.4	12.5	0.1715	36.4	29.7	0.7365
GI manifestations	18.2	4.2	0.2269	18.2	20.0	1.0000
Cardiac involvement	9.1	8.3	1.0000	9.1	8.6	1.0000
Renal crisis	9.1	0	0.3143	9.1	1.7	0.2180
Kidney involvement	9.1	0	0.3143	9.1	3.4	0.3521
Lung involvement	0	4.2	1.0000	0.0	20.0	0.1290
Interstitial lung disease	27.3	33.3	1.0000	27.3	73.1	**0.0030**
PAH	0	8.3	1.0000	0.0	17.1	0.2165
IgG, mean ± SD g/l	15.8 ± 6.2	14.3 ± 4	0.4191	15.8 ± 6.2	16.5 ± 6.3	0.7288
IgA, mean ± SD g/l	2.8 ± 0.9	2.4 ± 1.3	0.4034	2.8 ± 0.9	2.7 ± 1.3	0.8354
IgM, mean ± SD g/l	1.7 ± 0.6	1.7 ± 1.2	0.9001	1.7 ± 0.6	1.4 ± 0.8	0.3017
ESR, mean ± SD mm/h	17.6 ± 15.6	16.4 ± 17.4	0.8451	17.6 ± 15.6	23.4 ± 24.7	0.4442

* P values calculated using Pearson's chi-square or Fisher's exact test and 2-tailed T-test if necessary. CENP: centromere protein; ACA: anticentromere antibodies; GI: gastrointestinal; PAH: pulmonary arterial hypertension; ESR: erythrocyte sedimentation rate.

autoantigens for ACA. In addition, anti-CENP-P autoantibodies were also present in ACA-negative sera (6.0%).

In theory, a serum sample that can recognize a CENP should be able to stain the kinetochore and show an ACA-positive pattern in the IIF assay. However, we found that quite a few sera samples found to be ACA-negative by IIF were actually anti-CENP-B and/or -A positive by both commercial immunostrip assay and centromere protein microarray. Similarly, anti-CENP-P was found in IIF-negative sera. This discrepancy may suggest that either the IIF method is not sensitive enough, requiring higher antibody titer, or the CENP-P protein/epitopes are buried in the complex that blocks the access of their autoantibodies. On the other hand, 1 serum sample determined by IIF to be ACA-positive failed to recognize any of the 14 CENP in our microarray analysis as well as CENP-A and CENP-B by commercial immunostrips (supplementary data available from the author upon request), suggesting there might be additional autoantigens in the kinetochore yet to be discovered.

Historically, the presence of an ACA-associated staining pattern by IIF was a strong serological indicator for limited SSc or CREST syndrome[4], as well as a predictor of a more benign and protracted course for SSc[2]. It was also reported that ACA are much less frequently found in patients with ILD[2]. Similar results were obtained by surveying the 186 SSc serum samples in our study. Among the serologically ACA-positive patients, 82.9% (29/35) displayed a limited cutaneous form of SSc, and only 31.4% (11/35) of them were affected by ILD; whereas as many as 79.5% (120/151) of ACA-negative patients were diagnosed with the same diseases. The significantly reduced risk for ILD was also found in patients with anti-CENP-Q autoantibodies ($p < 0.01$) in our study.

Moreover, we discovered that patients with anti-CENP-P autoantibodies were more vulnerable to renal diseases in the ACA-negative cohort ($p = 0.042$), and even in the whole SSc cohort ($p = 0.0418$). The associations of anti-CENP-P antibodies with renal involvement are different from traditional clinical features associated with ACA[2]. This might suggest that anti-CENP-P antibody has unique clinical associations in SSc, especially in the setting of the ACA (negative) by traditional methods in the clinic. In addition, we found that anti-CENP-P did not coexist with anti-RNA polymerase, anti-U3-RNP, or anti-Th/To (data not shown), the autoantibodies for assessment of organ involvement in SSc. Therefore, anti-CENP-P may be a useful biomarker in clinical diagnosis of SSc.

Studies show that the prevalence of SSc-specific autoantibodies is different among various ethnic groups[38]. ACA appear to be much less frequent in some ethnic groups such as Thais (~2%), African Americans (~0%), and South African blacks (~0%)[2,39,40,41], but more frequent in whites (17%)[42], Danes (34%)[43], and white adults in the United States (27%)[44]. In our study, the samples were all from Chinese subjects. Therefore the usefulness of the newly identified CENP autoantigens in diagnosis of SSc for other groups needs further research.

In addition to CENP-P and -Q, 3 CENP antigens [CENP-J, -M (isoform I), and –T] were identified by microarray technology as new candidate ACA autoantigens, albeit at a lower occurrence. However, whether the autoantibodies against these 3 CENP are associated with specific clinical manifestations or laboratory features needs further analysis with larger cohorts. In our microarray, we included 2 isoforms for CENP-M, and only isoform I reacted with some ACA-positive sera. The 2 isoforms are identical in the first 102 amino acids, but differ greatly in their remaining sequences. Thus, the epitopes responsible for the autoimmunity of CENP-M must reside in the last 72 amino acids of isoform I.

Using a CENP-focused protein microarray, 5 CENP were identified as novel candidate ACA targets in SSc. Of them, CENP-P and -Q showed high sensitivities in ACA-positive sera. Subsequent validation analysis by ELISA and Western blot confirmed that both are ACA autoantigens. Additionally, anti-CENP-P autoantibodies were present in ACA-negative SSc sera at a lower rate. The presence of either anti-CENP-P or anti-CENP-Q was associated with some clinical manifestations and laboratory features.

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