Anti-cyclic citrullinated peptide antibody in systemic sclerosis

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Abstract

Objectives

To determine if anti-cyclic citrullinated peptide (anti-CCP) antibody titers can distinguish the overlap syndrome of systemic sclerosis and rheumatoid arthritis (SSc-RA) in patients with systemic sclerosis (SSc) and to investigate the clinical significance of anti-CCP antibodies in SSc.

Methods

Serum levels of anti-CCP antibodies were measured by enzyme-linked immunosorbent assay in 159 outpatients: 114 with SSc, 14 with rheumatoid arthritis, 7 with SSc-RA overlap syndrome, and 24 with Sjögren's syndrome. In patients with SSc and SSc-RA, we also measured serum levels of matrix metalloproteinase-3 and anti-agalactosyl IgG antibody.

Results

Elevated serum levels of anti-CCP antibodies were observed in 3 of 114 patients (2.6%) with SSc, 9 of 14 patients (64%) with RA, 6 of 7 patients (86%) with SSc-RA, and only 1 of 24 patients (4.2%) with SjS. In patients with SSc-RA, serum anti-CCP antibody levels were significantly higher than those seen in SSc ($p<0.001$). The sensitivity, specificity, and predictive values of elevated anti-CCP titers for SSc-RA were higher than either matrix metalloproteinase-3 and anti-agalactosyl IgG antibodies as markers. In addition, almost all SSc-RA and SSc patients with elevated serum levels of anti-CCP antibodies exhibited arthralgias and interstitial pneumonia.

Conclusions

Anti-CCP antibody titers are a reliable marker of SSc-RA facilitating its distinction from SSc alone.

Key words

Anti-cyclic citrullinated peptide antibody, overlap syndrome, rheumatoid arthritis, systemic sclerosis.

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This work was supported in part by a grant 15591174 from the Ministry of Education, Culture, Sports, Science and Technology of Japan (YoM) and the Foundation of Kyosaidan (YuM).

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Received on June 7, 2007; accepted in revised form on November 21, 2007.

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Competing interests: none declared.

Introduction

Systemic sclerosis (SSc) is characterized by thickening of the skin, fibrotic changes in the joints, muscles, and visceral organs, abnormalities in the microcirculation, and autoimmunity. Patients with SSc display a variety of clinical courses with a broad spectrum of severity of organ involvement. Musculoskeletal involvement is frequent in patients with SSc, clinically manifesting as arthralgias and/or arthritis, myalgias, muscle atrophy. Radiologically, these changes are seen as osteopenia, joint space narrowing, erosions, and subluxation (1-3). Montagna *et al*. determined that arthritis was present in approximately 13% (10/76) of SSc patients, with juxta-articular osteoporosis, joint space narrowing, and flexion contractures of the fingers frequently developing in the patient's hands (4). Patients with SSc often exhibit additional clinical features characteristic of rheumatoid arthritis (RA), defining the clinical condition of SSc-RA overlap syndrome. Horiki *et al*. reported that patients with SSc-RA display generalized skin sclerosis, severe seropositive polyarthritis, and pulmonary fibrosis, suggesting that SSc-RA may be a distinct entity from SSc alone (5). The majority of cases developed seropositive arthritis after onset of SSc. In the daily examination of SSc patients, it is important to identify those patients with overlapping RA who have greater functional impairment.

The diagnosis of RA primarily depends on the clinical manifestations of the disease. Although rheumatoid factor (RF) is detected in 60-80% of RA patients, the specificity of elevated RF is limited (48-92%), because RF is also detected in multiple non-RA rheumatic diseases, infectious diseases, and healthy individuals, particularly the elderly. Recent studies have utilized matrix metalloproteinase-3 (MMP3) and anti-agalactosyl IgG antibodies (AG-IgG) as markers of RA (6-8). Multiple autoantibodies in RA have emerged as useful markers for diagnosis and prognosis (9). Several studies revealed the clinical significance of autoantibodies against cyclic citrullinated peptide (CCP) in the diagnosis of RA (10-12). Elevated anti-CCP antibody titers are moderately sensitive (76%), but highly specific (96%) for RA (13). Anti-CCP antibodies are detectable in both seropositive and seronegative RA patients. Anti-CCP antibody levels also serve as a predictor of joint destruction (14-16). Thus, anti-CCP antibodies have become the most highly specific and predictive marker for the diagnosis of RA (17). In patients with SLE, erosive arthritis was strongly associated with the presence of anti-CCP antibodies (18).

This study sought to determine if anti-CCP antibody titers can distinguish SSc-RA from SSc alone. We also investigated the clinical significance of anti-CCP antibodies in these diseases.

Materials and methods

Patient selection

All patients were followed by the Department of Dermatology at Nagoya University School of Medicine. This study was comprised of 159 outpatients, 114 with systemic sclerosis (SSc), 14 with rheumatoid arthritis (RA), 7 with SSc-RA overlap syndrome (SSc-RA), and 24 with Sjögren's syndrome (SS). These 114 patients with SSc did not overlap with RA at the time of registration into the study. They were 144 females and 15 males, with ages ranging from 18 to 83 years and a mean age of 56 years (Table I). Patients with RA fulfilled the criteria proposed by the American College of Rheumatology (ACR) (19). All SSc patients fulfilled a new Japanese criterion, which was modified from the ACR criteria (20). The new criterion proposed a diagnosis of SSc in the presence of either the major criterion of proximal scleroderma or the minor criteria of sclerodactyly with one or more of the following criteria: 1) digital pitting scars on the fingertips or loss of substance of the distal finger pads, 2) bilateral basilar pulmonary fibrosis, or 3) a positive finding of anti-Scl-70 or anti-centromere antibody. The patients with SSc-RA were defined as those who fulfilled the criteria of both diseases. The sera were drawn from 1994 to 2006, after diagnosis of each disease. Especially, the sera of the patients with SSc-RA were all drawn after diagnosis of both diseases.

Patients diagnosed with SSc and SSc-

Table I. Patient profiles.

Disease	Number	Sex	Age	
		male : female	yrs	mean ± standard deviation
SSc	114	9 :105	19 - 83	58 ± 12
RA	14	2 : 12	35 - 76	56 ± 11
SSc-RA overlap	7	0 : 7	39 - 63	55 ± 8.0
SS	24	4 : 20	18 - 81	51 ± 17

SSc: systemic sclerosis; RA: rheumatoid arthritis; SS: Sjögren's syndrome.

RA were subcategorized into two subsets according to the classification system proposed by Leroy *et al*. (21); 34 exhibited diffuse cutaneous SSc (dSSc), while 87 had limited cutaneous SSc (lSSc). Patients with dSSc had a higher frequency of overlapping RA disease (4/34) than those with lSSc (3/87).
Interstitial pneumonia was assessed by chest CT scanning. Written informed consent was obtained from each subject, and this study was approved by the local Ethical Committee.

Antinuclear antibodies
Antinuclear antibodies (ANA) were detected by indirect immunofluorescence using HEp-2 cells (Fluoro HEPANA Test; MBL Co. Ltd., Nagoya, Japan) as previously described (22).

Rheumatoid factor
The latex agglutination test was used to detect RF with an RA-E kit (MBL Co. Ltd., Nagoya, Japan) (23). Briefly, a drop of latex reagent was added to serum samples on a slide and stirred with an applicator. The extent of agglutination was assessed after gently shaking the slide for one minute.

Anti-CCP autoantibodies
Serum antibodies directed against CCP were assessed by commercial enzyme-linked immunosorbent assay (ELISA) [DIASTAT™ Anti-CCP (Axis-Shield Diagnostics)] according to the manufacturer's instructions. In brief, 100 μl of anti-CCP standards (0, 2, 8, 30, and 100 U/ml) and pre-diluted (1:100) serum samples were distributed into individual wells. After a 60-minute incubation, wells were washed three times before incubation for 30 minutes with 100 μl of alkaline phosphatase-conjugated murine monoclonal antibody specific for human IgG. After three washes, 100 μl of chromogenic substrate solution was added to each well. The reaction was stopped after 30 minutes by the addition of sodium hydroxide-EDTA-carbonate buffer; the absorbance at 550 nm was then read using an ELISA plate reader (Multiskan JX, Thermo Electron Co. Ltd., USA). Serum samples were evaluated in duplicate. The above procedures were performed at room temperature. A cut-off value of 5.0 U/ml was recommended by ROC analysis performed at the manufacturer.

MMP-3
Serum levels of MMP-3 in patients with SSc and SSc-RA were measured using a commercial ELISA kit (Daiichi Fine Chemical, Toyama, Japan) according to the manufacturer's instructions. After pre-adsorbing anti-MMP-3 monoclonal antibody onto microtiter wells, 120 μl of peroxidase-conjugated anti-MMP-3 antibody was added to each well, followed by serum samples (40 μl) diluted to 1:4. After a 90-minute incubation followed by four washes, color was developed with hydrogen peroxide and tetramethylbenzidine peroxidase. The reaction was stopped with vitriolic acid after a 30-minute incubation; the absorbance at 450 nm was then measured. The concentration of MMP-3 in each sample was determined by comparison to a standard curve. Values greater than the 95% confidence interval for normal control subjects were designated as elevated. The normal ranges for MMP-3 were 36.9~121 ng/ml for males and 17.3~59.7 ng/ml for females, according to the manufacturer's data.

Anti-agalactosyl IgG antibodies
Serum levels of anti-agalactosyl IgG antibodies (AG-IgG) in patients with SSc and SSc-RA were measured using a commercial lectin-enzyme immunoassay kit (Eitest CARF; Eisai Co. Ltd., Tokyo, Japan) (24). Briefly, serum samples (100 μl) diluted 201-fold were added to wells coated with agalactosyl IgG. After a one-hour incubation, wells were washed three times. Next, 100 μl biotinylated lectin (Ricinus communis agglutinin) was added. After a one-hour incubation, wells were washed three times before the addition of 100 μl of streptavidin-peroxidase conjugate solution. After a one-hour incubation followed by three washes, 100 μl chromogen substrate solution was added. The reaction was stopped by the addition of 2 mM sodium azide after a 30-minute incubation; the absorbance at 405 nm was then read using an ELISA plate reader. The upper limit of the reference range was set at 6.0AU/ml by ROC analysis performed at the manufacturer.

Statistical analysis
Statistical analysis utilized the Mann-Whitney U-test for comparison of means. Fisher's exact probability test was used to analyze frequencies. Statistical procedures were performed using SPSS Ver.13 statistical software from SPSS Japan, Inc. A p-value less than 0.05 was considered to be statistically significant.

Results

Serum levels of anti-CCP antibodies
Figure 1 displays the serum levels of anti-CCP antibodies in patients with SSc, RA, SSc-RA, and SS. Antibody levels in patients with SSc-RA were significantly elevated in comparison with those seen in SSc patients (mean ± SD: 50.81±67.40 *vs*. 2.10±12.50U/ml, $p<0.001$). Patients with RA had significantly higher antibody titers than those with SSc (mean ± SD: 64.58±90.34 *vs*. 2.10±12.50U/ml, $p<0.001$). There were no significant differences between antibody levels in patients with RA and SSc-RA ($p=0.628$). All SS patients exhibited low antibody titers, with a mean of 0.89±3.01 U/ml (mean ± SD). When the cut-off value was set at 5U/ml, elevated antibody levels were only observed in 3 of 114 patients (2.6%) with SSc, 9 of 14 patients (64%) with RA, 6 of 7 patients (86%) with SSc-

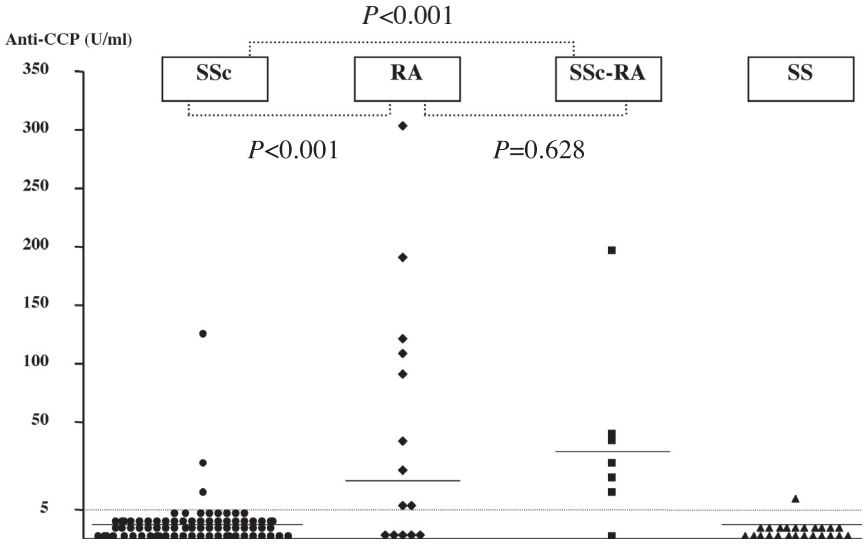

Fig. 1. Serum levels of anti-CCP antibodies. Bars indicate medians of the data. The horizontal dotted line shows the cut-off value (5U/ml). Comparisons between diseases were made using the Mann-Whitney U test. *P*-values less than 0.05 were considered to be significant.

RA, and 1 of 24 patients (4.2%) with SS. Six patients with SSc-RA, who demonstrated elevated serum levels of anti-CCP antibodies, also possessed RF, while another patient with SSc-RA lacked both anti-CCP antibody and RF.

Clinical features in SSc and SSc-RA patients with elevated serum levels of anti-CCP antibodies

The clinical and laboratory features of the SSc and SSc-RA patients with elevated serum levels of anti-CCP antibodies are shown in Table II. Six patients had dSSc and three had lSSc. All nine of these patients had arthralgias and positive ANA titers. Eight patients had evidence of interstitial pneumonia confirmed by computed tomography and exhibited positive RF titers and elevations of AG-IgG. Their pattern on chest CT demonstrated predominantly dorsal and lower zone honeycombing on a background of ground-glass attenuation. Seven patients were positive for MMP-3. Notably, one patient with SSc (No. 3 in Table II) developed RA nine years after serum examination.

In patients with normal anti-CCP antibody levels, 21% (23/107) and 36% (38/107) developed arthralgias and interstitial pneumonia, respectively. Fisher's exact probability test revealed a statistically significant association of elevated serum levels of the anti-CCP antibodies with arthralgias ($p<0.001$) and interstitial pneumonia ($p<0.005$).

Serum levels of MMP-3 and anti-agalactosyl IgG antibodies

Elevated serum MMP-3 levels were observed in 20 of 114 patients (18%) with SSc and 5 of 7 patients (71%) with SSc-RA when cut-off levels were set at 36.9-121 ng/ml and 17.3-59.7 ng/ml for males and females, respectively. Twenty-two females and three males were seropositive for MMP-3, with ages ranging from 39 to 83 years and a mean of 60 years.

In addition, 23 out of 114 patients (20%) with SSc and 5 out of 7 patients (71%) with SSc-RA were positive for AG-IgG when the upper limit of the reference range was set to 6.0 AU/ml. Twenty-six females and two males were seropositive for AG-IgG; the ages

Table II. Clinical and laboratory features in nine patients with SSc and SSc-RA overlap syndrome with elevated serum levels for anti-cyclic citrullinated peptide antibodies.

No.	Age	Sex	Disease	Preceding disease	Interval (years)*	Usage of PSL (mg/day)	arthralgia	IP	CRP (mg/dl)	RF	ANA	CCP (U/ml)	MMP3 (ng/ml)	AG IgG (AU/ml)
1	50	M	dSSc			17.5	+	+	0	+	SS-A	31	195	240
2	75	F	dSSc			7.5	+	+	0.44	-	Topo-1	15	290	8
3	68	F	dSSc#			0	+	+	0	+	Topo-1	130	9	6.2
4	52	F	lSSc, RA	SSc	8	5	+	+	3.4	2+	ACA	22	155	370
5	39	F	dSSc, RA	SSc	5	4	+	+	0.1	2+	Topo-1	18	245	120
6	63	F	lSSc, RA	SSc	9	5	+	-	2.9	2+	ACA	200	480	4.3
7	58	F	dSSc, RA	RA	14	6	+	+	8.5	2+	Topo-1	30	82	200
8	56	F	lSSc, RA	SSc	11	10	+	+	2.91	+	Topo-1	40	110	26
9	62	F	dSSc, RA	SSc	2	10	+	+	0.72	+	Nuclear speckled and cytoplasmic	45	35	25.5

SSc: systemic sclerosis; lSSc: limited cutaneous SSc; dSSc: diffuse cutaneous SSc; RA: rheumatoid arthritis; PSL: prednisolone; IP: interstitial pneumonia; CRP: C-reactive protein; RF: rheumatoid factor; ANA: antinuclear antibodies; SS-A: anti-SS-A antibody; Topo-1: anti-topoisomerase 1 antibody; ACA: anti-centromere antibody; CCP: serum levels of anti-cyclic citrullinated peptide antibody; MMP-3: serum levels of matrix metalloproteinase-3; AG-IgG: serum levels of anti-agalactosyl IgG antibodies.

*Interval between diagnoses with the two diseases

#This patient developed RA nine years after serum examination.

Table III. Diagnostic characteristics for SSc-RA overlap syndrome.

Seropositive to	CCP	MMP-3	AG-IgG	MMP-3 and/or AG-IgG	CCP and/or MMP-3	CCP and/or AG-IgG
Sensitivity (%)	86	71	71	86	86	86
Specificity (%)	97	82	80	68	82	80
PPV (%)	67	20	18	14	22	21
NPV (%)	99	98	98	99	99	99

CCP: anti- cyclic citrullinated peptide antibody; MMP-3: matrix metalloproteinase-3; AG-IgG: anti-agalactosyl IgG antibodies; PPV: positive predictive value; NPV: negative predictive value.

of these patients ranged from 39 to 83 years with a mean of 60 years.

Diagnostic characteristics for SSc-RA

The diagnostic characteristics for SSc-RA are detailed in Table III. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for SSc-RA of elevated anti-CCP antibodies were 86%, 97%, 67%, and 99%, respectively. In comparison to elevated titers of MMP-3 and/or AG-IgG as diagnostic parameters, elevated titers of anti-CCP antibodies demonstrated better performance for the diagnosis of SSc-RA.

Discussion

Patients with SSc frequently demonstrate musculoskeletal involvement, which manifests as arthralgias, arthritis, myalgias, and muscle atrophy. A subset of SSc cases, however, exhibits the clinical characteristics of RA, dubbed overlap syndrome (25-26); these patients exhibit more severe arthralgias and functional impairment than patients with SSc alone (5). In their description of 15 SSc-RA patients, Horiki *et al*. reported that all patients had erosive polyarthritis and RF seropositivity, 87% (13/15) exhibited pulmonary fibrosis, and 80% (12/15) had proximal scleroderma. Doran *et al*. also described four patients with SSc-RA, all of whom had erosive lesions on hand radiographs (27). All of the patients had pulmonary fibrosis.

A previous investigation utilizing the Health Assessment Questionnaire (HAQ) clarified that patients suffering from SSc-RA exhibited greater functional impairments than patients with SSc alone, as evidenced by a higher HAQ-disability index for patients with SSc-RA than that determined for patients with SSc (28, our unpublished observations). To distinguish those patients with SSc-RA from the entirety of SSc patients, identification of reliable disease markers for SSc-RA is necessary, which could allow the early detection and rapid treatment of the disease. In this study, we evaluated the utility of elevated anti-CCP antibody titers in the distinction of SSc-RA from SSc alone. We also investigated the utility of anti-CCP antibody measurement in the evaluation of patients with SSc in comparison to MMP-3 and AG-IgG, two accepted serological markers for RA.

Our results demonstrated that the seroprevalence of anti-CCP antibodies was significantly higher (86%) in patients with SSc-RA than that seen in patients with SSc alone (2.6%). Ingegnoli *et al*. observed anti-CCP antibodies in 10.6% (8/75) of SSc patients (29), which was a higher proportion than seen in our data set. As they mentioned, the population of SSc patients that they examined may have included SSc-RA patients. Thus, the seroprevalence of antibodies in patients with SSc alone may be significantly lower than 10.6%. Anti-CCP antibodies are a superior serological marker to MMP-3 and AG-IgG, allowing for more accurate diagnosis of SSc-RA; the sensitivity, specificity, PPV, and NPV of elevated anti-CCP antibodies for SSc-RA diagnosis were higher than those seen for MMP-3 and AG-IgG (Table III). Recently, Szücs *et al*. published a report detailing SSc-RA overlap syndrome. They detected anti-CCP antibodies in 18/22 patients (82%) with SSc-RA (30). In this study, we confirmed high positivity for anti-CCP antibody in Japanese patients with SSc-RA.

Our data might imply that anti-CCP antibodies also correlate with joint involvement and interstitial pneumonia in SSc. In most SSc patients with elevated serum levels of anti-CCP antibodies, they overlapped with RA and were classified into dSSc rather than lSSc. As patients with RA have arthralgias and patients with dSSc often have interstitial pneumonia, the relationship between anti-CCP antibody titers and arthralgias/interstitial pneumonia may be result from overlapping with RA and/or subset of SSc. To clarify the association between anti-CCP antibodies and both pulmonary disease and joint involvement, it will be necessary to examine larger cohorts; this study included too few patients to discuss the correlations.

In conclusion, anti-CCP antibody titers distinguish SSc-RA from SSc alone with a high specificity. We recommend measuring anti-CCP antibody titers when examining SSc patients suffering from joint involvement to facilitate the early detection and intervention of SSc-RA. In five of the six patients with SSc-RA who were positive for anti-CCP antibodies, SSc preceded RA. One SSc patient with high anti-CCP antibody titers developed RA nine years after serum testing. We are currently following the remaining two anti-CCP-positive patients with SSc alone for the development of RA.

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