Anti-centromere antibodies target centromere–kinetochore macrocomplex: a comprehensive autoantigen profiling

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ABSTRACT

Objectives Anti-centromere antibodies (ACAs) are detected in patients with various autoimmune diseases such as Sjögren’s syndrome (SS), systemic sclerosis (SSc) and primary biliary cholangitis (PBC). However, the targeted antigens of ACAs are not fully elucidated despite the accumulating understanding of the molecular structure of the centromere. The aim of this study was to comprehensively reveal the autoantigenicity of centromere proteins.

Methods A centromere antigen library including 16 principal subcomplexes composed of 41 centromere proteins was constructed. Centromere protein/complex binding beads were used to detect serum ACAs in patients with SS, SSc and PBC. ACA-secreting cells in salivary glands obtained from patients with SS were detected with green fluorescent protein-fusion centromere antigens and semiquantified with confocal microscopy.

Results A total of 241 individuals with SS, SSc or PBC were recruited for ACA profiling. A broad spectrum of serum autoantibodies was observed, and some of them had comparative frequency as anti-CENP-B antibody, which is the known major ACA. The prevalence of each antibody was shared across the three diseases. Immunostaining of SS salivary glands showed the accumulation of antibody-secreting cells (ASCs) specific for kinetochore, which is a part of the centromere, whereas little reactivity against CENP-B was seen.

Conclusions We demonstrated that serum autoantibodies target the centromere–kinetochore macrocomplex in patients with SS, SSc and PBC. The specificity of ASCs in SS salivary glands suggests kinetochore complex-driven autoantibody selection, providing insight into the underlying mechanism of ACA acquisition.

INTRODUCTION

Anti-centromere antibodies (ACAs) are well-known autoantibodies detected in various autoimmune diseases. Although serum ACAs are frequently detected in patients with systemic sclerosis (SSc), they are also detected in other autoimmune diseases such as Sjögren’s syndrome (SS) and primary biliary cholangitis (PBC), and the presence of ACAs is associated with the overlap of these three diseases.1–3

In the anti-nuclear antibody (ANA) test, ACAs show a characteristic staining pattern called the discrete-speckled pattern, which reflects the localisation of the centromere.4 Recently, the molecular structure of the centromere has been rapidly clarified. Its framework structure is understood as a combination of specific centromeric chromatin, characterised by the replacement of histone H3 by CENP-A and the macromolecular complex ‘kinetochore’, which is assembled on the centromere-specific nucleosome.5 The centromere binds to microtubules via inner and outer kinetochore structure during cell division. Schematic illustration of

Key messages

What is already known about this subject?

► Anti-centromere antibodies (ACAs) are detected in various autoimmune diseases such as Sjögren’s syndrome (SS), systemic sclerosis (SSc) and primary biliary cholangitis (PBC) and correlate with characteristic symptoms such as Raynaud’s phenomenon and sclerodactyly.

What does this study add?

► Comprehensive serum ACA profiling revealed broad specificity for the centromere–kinetochore macrocomplex, and the specificity of autoantibodies was not different in patients with SS, SSc and PBC.

► Antibody-secreting cells in the salivary glands of ACA-positive SS patients were specific to the part of the centromeric structure, termed the 'kinetochore' rather than CENP-B, which is known as the major autoantigen corresponding to ACA.

How might this impact on clinical practice or future developments?

► This study conducted a detailed analysis of the specificity of ACAs, providing further insights into pathogenic autoantibodies common to multiple autoimmune diseases.

► The combination of multiple conformational centromere antibodies could detect serum ACAs with higher sensitivity than conventional ACA detection methods.
Autoimmunity

Figure 1 Schematic illustration of the centromere–kinetochore–microtubule interface. CENP-A replaces histone H3 and forms centromere-specific nucleosome. CBX5 and CENP-B bind to H3 nucleosome and centromeric DNA, respectively. The kinetochore complex is constructed on the CENP-A nucleosome and interacts with microtubules. The key kinetochore subcomplexes are the constitutive centromere-associated network (CCAN; divided into CENP-C, CENP-HIKM, CENP-TWSX, CENP-LN, and CENP-OPQR) and the KMN-network (divided into the KNL1 complex, the MIS12 complex, and the NDC80 complex). The Astrin-SKAP complex and the Ska1 complex stabilise the kinetochore-microtubule binding. *Known autoantigens in autoimmune diseases.12 15 17

Table 1 Clinical characteristics of the patients who underwent serum analysis

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<th>Disease type/ complicated disease</th>
<th>HC n=68</th>
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<th>SSc n=35</th>
<th>PBC n=10</th>
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ACA, anti-centromere antibody; AMA, anti-mitochondrial antibody; dSc5c, diffuse cutaneous systemic sclerosis; HC, healthy controls; lSc5c, limited cutaneous systemic sclerosis; NA, not assessed; PBC, primary biliary cholangitis; RNAPIII, RNA polymerase III; SS, Sjögren’s syndrome; SSc, systemic sclerosis; Topo 1, topoisomerase 1.
several studies have focused on the distinct epitope specificity of major antigens (ie, CENP-A, -B and -C), comparing patients with SS and SSc,

Our recent study of autoantibodies produced in salivary glands demonstrated that many autoantibodies recognise native conformational epitopes. We developed an antigen-binding bead assay by using mammalian cell line-derived proteins.
which provided higher sensitivity for the detection of autoantibodies than conventional ELISA. In this study, we constructed a centromere antigen library including 41 centromere proteins, which were selected based on the latest information about the centromere structure. To clarify the true target of ACAs and to identify differences by disease, we examined serum autoantibodies against this library using an antigen-binding bead assay and investigated the spatial relationship of antibody-secreting cells (ASCs) in salivary gland tissue.

METHODS

Clinical samples
Serum samples were obtained from patients with SS, SSc or PBC, and salivary gland samples were collected from patients with clinically suspected SS who underwent a lip biopsy at Keio University Hospital. The diagnosis was made according to the 2016 American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) classification criteria for primary SS, the 2013 ACR/EULAR classification criteria for SSc and the clinical practice guidelines for PBC established in 2017. Sera of healthy controls (HCs) were used as controls.

Preparation of the centromere antigen library
A total of 41 centromere proteins were cloned into pEFs vector or pcDNA3.4 vector as a centromere protein library, combined with the streptavidin-binding peptide tag and green fluorescent protein (GFP) at the N-terminus and expressed by 293T cells. Most of them were cotransfected to construct subcomplexes according to the molecular structure of the centromere. Antigens were purified by streptavidin beads and electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by silver staining (Aproscience, Tokushima, Japan) and western blotting with anti-human GFP antibody (unconjugated, 1GFP63, BioLegend, California, USA) and horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (GE Healthcare, Buckinghamshire, UK) (see online supplemental figure S1). Nucleic acid of CENP-E was purchased from Kazusa DNA Research Institute (Chiba, Japan). Other detailed methods were described previously.

Serum autoantibody detection by antigen-binding bead assay
The protocols for bead coupling and measurements of serum antibody titres were described previously. In short, antigens expressed by 293T cells were attached to Dynabeads M-280 Streptavidin (Thermo Fisher Scientific, Massachusetts, USA). Antigen-binding beads were incubated with sera of subjects, washed and then stained with anti-human IgG-Fc antibody (APC, goat-F(ab')2, fragment, Jackson ImmunoResearch, Pennsylvania, USA) and anti-human IgA-Fc antibody (DL405, goat-F(ab')2 fragment, Jackson ImmunoResearch). The titres of antibodies were analysed by FACSVerse and FlowJo software (BD Biosciences, California, USA). Anti-CENP-B antibody was measured by anti-CENP-B ELISA (ORGENTEC, Mainz, Germany) according to the manufacturer’s instructions.

Monoclonal antibodies against newly identified centromere autoantigens
We previously examined lesion antibody specificity by cloning antibodies from human salivary glands and analysed their reactivity to recombinant centromere antigens. In this study, these cloned antibodies were comprehensively analysed with a newly developed centromere antigen library. The detailed method is described in online supplemental information.

Direct detection of antibody-producing cells in salivary glands
Fresh-frozen sections of labial salivary gland samples were incubated with GFP fusion antigens and anti-CD138 antibody and anti-mouse IgG1 antibody. ASCs were semiquantified using confocal microscopy. The detailed method is provided in online supplemental information.

Statistics
The cut-off value of a serum antibody against a specific antigen was determined by the median plus 1.5 IQR of the mean fluorescent intensity in 68 HCs. A doubling of the difference between the third quartile and the median (Q3−Q2) was substituted for IQR when the skewness of a distribution in HCs was over
Autoimmunity

1.24 Exceptionally, the cut-off value of anti-CENP-B IgG antibody was determined by receiver operating characteristic analysis, in which patients with positive anti-CENP-B antibody by ELISA were defined as positive. The Wilcoxon rank sum test was applied to compare continuous variables, and two-sided Fisher’s exact test was applied to compare categorical variables. P values <0.05 were considered statistically significant. Unsupervised hierarchical clustering by Ward’s method and principal component analysis (PCA) were performed to analyse the serum autoantibody profile. GraphPad Prism 8 (GraphPad Software, California, USA) and JMP V.13 (SAS Institute, North Carolina, USA) were used to perform the analyses.

RESULTS

Serum anti-centromere antibody analysis

We recruited a total of 241 individuals with SS (n=86), SSc (n=35), PBC (n=10) or two or more diseases above (overlap; n=42) and HC (n=68) for serum antibody analysis. The clinical characteristics of the subjects are shown in table 1.

The IgG reactivity of each individual sera against centromere antigens is shown in figure 2A. The reactivity against CENP-HIKM, CENP-TWSX, CENP-OPQUR and CENP-LN was observed with a similar tendency to those against previously known autoantigens: CBX5, CENP-A, CENP-B, CENP-C and the MIS12 complex (MIS12C). Reactivity against the NDC80 complex (NDC80C), the KNL1 complex (KNL1C), the Astrin–SKAP complex, CENP-E and CENP-F was also seen with relatively low frequency. The Rod-Zw10-Zwilch (RZZ) complex and the Ska1 complex showed negligibly low reactivity in the sera. Although we also performed the same analysis with IgA antibodies, the titres and antibody positivity were low, and the differences between the HC group and each disease group were less apparent than those of IgG antibodies (online supplemental figure S2).

As shown in figure 2B, the positive rates of antibodies against CENP-HIKM, CENP-TWSX, CENP-OPQUR, CENP-LN, NDC80C, KNL1C and the Astrin–SKAP complex were significantly higher in at least one disease group than in the HC group. The differences between the HC group and each disease group were less apparent than those of IgG antibodies (online supplemental figure S2).

As shown in figure 2B, the positive rates of antibodies against CENP-HIKM, CENP-TWSX, CENP-OPQUR, CENP-LN, NDC80C, KNL1C and the Astrin–SKAP complex were significantly higher in at least one disease group than in the HC group. The differences between the HC group and each disease group were less apparent than those of IgG antibodies (online supplemental figure S2).

Clustering of autoantigens and individuals

Next, we performed clustering analysis to visualise the serum autoantibody profile of each individual and analysed the correlations among antigens. Unsupervised hierarchical clustering identified two antigen clusters (figure 3). We refer to the first cluster, including CBX5, CENP-A, CENP-B, CENP-C, CENP-HIKM, CENP-TWSX, CENP-OPQUR, CENP-LN, and MIS12C, as the ‘major antigen’ cluster and the second cluster, including the
against centromere proteins were well correlated with their titres by ELISA. As shown in figure 6.


Next, we analysed the clinical significance of comprehensive results indicated that the specificity of ACAs is generally shared in these diseases (online supplemental figure S3B). These overlap groups, suggesting similar patterns of antibody specificity in these diseases (online supplemental figure S3B). These results indicated that the specificity of ACAs is generally shared across disease phenotypes.

Antigen bead assay identified potential ACA-positive patients

Table 2 Clinical characteristics and antibody specificity of lesion antibody-secreting cells in salivary glands

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Slides were examined at a magnification of 200×. -, undetectable; ±, one cell in multiple fields; +, 1–3 cells in one field; ++, 4–8 cells in one field; ++++,>8 cells in one field.

The patient IDs correspond to those in our previous study.17

*ACA converted to positive after 1 year of immunosuppressive therapy.
†Treated with prednisolone 10 mg/day.
C, cytoplasm; D, discrete-speckled; F, female; H, homogenous; LB, lip biopsy; MCTD, mixed connective tissue disease; N, nucleolar; NA, not assessed; PH, pulmonary hypertension; PMR, polymyalgia rheumatica; pSS, primary Sjögren’s syndrome; RZZ, Rod-Zw10-Zwilch; S, salivary gland; Sp, speckled; SS, Sjögren’s syndrome; sSS, secondary Sjögren’s syndrome.

Others, as the ‘minor antigen’ cluster. In the major autoantigen cluster, the titre of each antibody was mutually correlated in addition to the prevalence (online supplemental figure S3A).

When we focused on the disease status, the participants seemed to be classified into three groups. A total of 54 individuals in cluster A showed a broad spectrum of autoantibodies against the major antigens. For the remaining participants, cluster B, with 50 individuals, had 1–7 antibodies against major or minor antigens, whereas cluster C, with 137 individuals, had few or none. Although patients with overlapping diseases tended to be classified as cluster A and most HCs were in cluster C, the result of clustering based on the antibody profile was not consistent with disease specificity. We further performed PCA of serum IgG antibody reactivity and revealed overlapping 95% confidence ellipses across patients in the SS, SSc, PBC and overlap groups, suggesting similar patterns of antibody specificity in these diseases (online supplemental figure S3B). These results indicated that the specificity of ACAs is generally shared across disease phenotypes.

Anti-CENP-B antibody by ELISA (r²=0.6190, p<0.0001). The presence of a discrete-speckled pattern by the ANA test was remarkably consistent with the positivity of the anti-CENP-B antibody by ELISA.

Although most HCs have no more than one antibody, some patients without ACA by standard ACA detection methods have two or more antibodies. Defining the bead assay positivity as having two or more antibodies, the bead assay clearly distinguished patients with SS, SSc or PBC with high specificity (figure 4A, online supplemental table S1). As shown in figure 4C, the bead assay identified 14%–23% of additional patients as ACA positive compared with standard methods in each disease group (15% in SS, 23% in SSc, 20% in PBC and 14% in overlap).

To confirm the clinical significance of additionally identified ACAs, we compared the characteristics of patients with or without anti-CENP-B antibody. The clinical characteristics of bead assay-positive SS patients with or without anti-CENP-B antibody by ELISA were comparable between the two groups (online supplemental table S2). For patients with SSc, the clinical characteristics differed in the two groups (online supplemental table S3). Although all anti-CENP-B antibody-positive patients presented limited cutaneous SSc, half of the bead assay-positive and ELISA-negative patients showed diffuse cutaneous SSc with anti-topoisomerase 1 antibody (ATA). The number of patients with PBC was too small to compare clinical characteristics (online supplemental table S4).

Monoclonal antibodies from SS salivary glands recognised the newly identified centromere antigens

In a previous study, we produced 256 antibodies from the ASCs of SS salivary glands, some of which were reacted against known centromere autoantigens. In this study, we searched for antibodies that recognise new centromere autoantigens. Antibodies against CENP-C, CENP-HIKM, CENP-OPQUR, and the MIS12 complex were secreted in the same area of salivary glands from distinct ASCs. Scale bar: 100 μm. ASCs, antibody-secreting cells; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein.

Antigen specificity of ASCs in salivary glands

Next, we comprehensively analysed the specificity of ASCs in target organs using a centromere antigen library. Due to limitations in sample collection, the study was limited to salivary glands in patients with SS. Fresh-frozen sections of labial salivary glands were stained with GFP-fusion centromere antigens and CD138 as a cell surface marker of ASCs. Representative images are shown in online supplemental figure S4.

The results are summarised in table 2. Among ACA-positive patients (n=8), 6 patients had lesion ASCs targeting centromere antigens. The most frequent target antigens were CENP-C (n=6) and MIS12C (n=6), followed by CENP-HIKM (n=4), NDC80C (n=3) and CENP-OPQUR (n=2). ASCs targeting other major antigens were scarce or not identified in salivary glands. ASCs in patients with ACA-negative SS (n=5) and patients with sicca symptoms without fulfilling SS diagnosis (n=2) showed negative results. These results were consistent with the results of the specificity of monoclonal antibodies from ACA-positive SS salivary glands.

In addition, an analysis of serial sections demonstrated the distribution of ASCs against various kinetochore antigens (CENP-C, CENP-HIKM, CENP-OPQUR, and the MIS12 complex) in the same area of salivary glands from distinct ASCs. Scale bar: 100 μm. ASCs, antibody-secreting cells; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein.

DISCUSSION

We provided the comprehensive mapping of ACAs targets within autoimmune diseases. We demonstrated that the CCAN was the major target of serum autoantibodies as well as the previously known autoantigens CBX5, CENP-A, CENP-B, CENP-C and MIS12C in patients with SS, SSc and PBC. These results
indicated that the centromere–kinetochore macromolecular complex is the main target of serum ACAs. In addition, the autoantigencity of centromere antigens was shared among patients with SS, SSc and PBC. With regards to the ASCs in SS salivary glands, kinetochore antigens, rather than centromeric proteins such as CENP-B, were the dominant targets of ASCs as opposed to serum ACAs.

Several studies indicated that ACA may cross-react with some non-centromere proteins. Although we could not rule out cross-reactivity with non-centromere proteins, however, we found that when an autoantibody is acquired against at least one major centromere antigen, it is likely to be accompanied by multiple ACAs as shown in figure 3. These results could not be explained by molecular mimicry alone and suggest that ACAs would recognise the structure of the centromere complex, rather than a single epitope.

Although a previous study showed that serum ACAs had relatively low or no reactivity against centromere proteins other than CENP-A, CENP-B and CENP-C, their reactivity might be underestimated due to the usage of non-mammalian cell-derived individual proteins regardless of their intermolecular association. We previously reported that using human cell-derived antigens and coexpression of the complexed proteins enables highly sensitive detection of autoantibody. Accumulating evidence about the molecular structure of the centromere enabled us to build a centromere antigen library in which the intermolecular conformation was taken into account. In this study, we identified multiple novel targets of ACAs, such as CENP-HIKM, CENP-TWSX, CENP-OPQR, CENP-LN, NDC80C, KNL1C and the Astrin–SKAP complex. We believe that this concept, using conformational antigens for antibody detection, could be applicable to identify novel autoantibodies in other autoimmune conditions.

Although several studies have focused on the distinct epitope specificity comparing patients with SS and SSc, demonstrating that antibodies against CBX5 and CENP-C are frequently seen in SS compared with SSc, our present study clarified the similarity in serum autoantibodies against nine major autoantigens. Several reports demonstrated the frequent concurrence of SS/SSc, SSc/PBC and SSc/PBC in the presence of ACAs. Moreover, the presence of ACAs is associated with characteristic symptoms such as Raynaud’s phenomenon, sclerodactyly and sicca syndrome regardless of whether classification criteria are fulfilled. Taken together, these results indicate that patients with ACA-positive SS, SSc and PBC have common clinical and immunological characteristics, strengthening our idea that novel disease classification, ‘ACA-related disease’, could be added to the disease category.

We further demonstrated the potential for the clinical application of the assay with multiple centromere antigens. Previous studies showed that the ELISA results of the anti-CENP-B antibody highly corresponded with the discrete-speckled pattern by the ANA test; hence, these principal 2 methods could only detect the common population. Our data demonstrated that 14%–23% of the patients with SS, SSc or PBC had autoantibodies against multiple centromere antigens but not CENP-B. Furthermore, these antibodies were highly specific to autoimmune diseases. Additionally identified ACA positivity might have comparable clinical significance to anti-CENP-B antibody in patients with SS because the clinical characteristics were similar regardless of the presence of anti-CENP-B antibody. In SSc patients, although the clinical characteristics of ACA-positive patients with or without anti-CENP-B antibody were different in accordance with the prevalence of ATA, this result was consistent with that of previous reports that ATA and ACA were not mutually exclusive, and the clinical manifestations of ATA and ACA double-positive patients were similar to those of ATA single-positive patients.

ASCs in the salivary glands of ACA-positive patients with SS showed reactivity to various centromere antigens and characterised by specificity to kinetochore antigens. There is accumulating evidence of local antibody production in target organs of systemic autoimmune diseases. In SS, several studies described the antigen-driven immune response and autoantibody production from B cells within salivary glands. In our study, the observed diversity of ASCs against kinetochore proteins corroborated the presence of serum various ACAs in autoimmune diseases. Although serum autoantibodies showed similar reactivity against major autoantigens, including both centromeric chromatin and kinetochore proteins, ASCs in the salivary glands showed specificity to kinetochore proteins. In addition, immunostaining of serial sections with various kinetochore antigens showed the local accumulation of distinct anti-kinetochore ASCs. These results suggested that the kinetochore complex is presented to B cells and causes kinetochore-driven antibody selection within salivadentis in SS.

We note that this study has several limitations. The diagnostic potential of our method should be verified in a large cohort of seronegative autoimmune disease patients because many of the patients in our cohort were already diagnosed with conventional disease-specific autoantibodies. In addition, our result might not reflect the serology in early stage of each disease because the patients, especially in SSc group, had relatively long disease duration. Moreover, the result from ASCs of SS salivary glands could show the indirect evidence of kinetochore-driven antibody selection, however, further research is needed to clarify the precise mechanism of ACA acquisition. The specificity of ASCs in affected organs other than SS salivary glands remains a challenge due to the limitation of sample collection.

In conclusion, our study presented the precise mapping of ACA targets, indicating that the centromere–kinetochore macromolecular complex is the main target of serum ACAs and that patients with ACA-positive SS, SSc and PBC form distinct subgroups in terms of the similarity of antibody specificity. The acquisition of ACAs might be the result of kinetochore complex-driven antibody selection in affected organs.

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Contributors Study design: NK, MT, KS, KT and TT. Data acquisition: NK, MT, YK, HY, KI, HS, SK, HS and KT. Data analysis and interpretation: NK, MT and KS. Manuscript drafting: NK, MT, KS and TT. All authors approved the final version of the manuscript.

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Competing interests MT, KS, and TT have applied for a patent of anti-MIS12C antibody as diagnostic marker.

Patient consent for publication Not required.

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