The importance of specific citrullinated clusterin and vimentin found in a multi-coloured bead-based citrulline-peptide array system in rheumatoid arthritis

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Abstract

Objective

The importance of citrullination in rheumatoid arthritis (RA) has been reported, but the degree to which individual citrullinated proteins affect the onset and progression of RA is still unclear. We aimed to identify citrullinated proteins that may play an important role in the onset and progression of RA using an individualised anti-citrullinated protein antibody (ACPA) evaluation system with citrullinated peptides as probes.

Methods

Serum samples from 50 normal donors and 51 RA patients were evaluated using a custom MagPlex™ bead array with 13 types of citrullinated peptide. The presence/absence of ACPAs that react to each citrullinated peptide in each subject was determined using the Z-score, which was calculated based on the fluorescence intensity distribution of a sample from a normal donor. Whether the fluorescence intensity was inhibited when free citrullinated peptides were added to a system was also evaluated.

Results

Median fluorescence intensities obtained from beads coupled with the 13 types of citrullinated peptide were all significantly higher in RA patients versus normal donors. With a Z-score ≥2 as the cut-off value for the presence of ACPAs, ACPAs that recognised five types of citrullinated peptides derived from fibrinogen A, fillagrin, clusterin, and vimentin were widely detected in RA patients. In addition, inhibition experiments showed that citrullinated vimentin, clusterin, and enolase 1A peptides inhibited coupling of ACPAs to other citrullinated peptides.

Conclusion

ACPAs to many citrullinated proteins exhibited cross-reactivity to citrullinated clusterin and vimentin, suggesting the importance of citrullinated clusterin and vimentin in the early stages of RA pathogenesis.

Key words

anti-citrullinated protein antibodies, citrullination, clusterin, protein array assay, rheumatoid arthritis, vimentin

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease characterised by generalised arthritis and synovitis of unknown aetiology (1, 2). Several cellular components, soluble mediators, adhesion molecules, and autoantibodies are thought to play a role in the pathogenesis of this disease (3). RA is clinically characterised by the presence of autoantibodies including rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPAs) (4). Citrullination is the post-translational modification of arginine to citrulline, and citrullinated proteins are typically found in the synovium of inflamed joints as well as in the lungs of RA patients (1, 2, 4-6).

The autoimmune response against anti-citrullinated protein has been hypothesised to contribute to the pathogenesis of RA (1-8). ACPAs have been detected up to 10 years prior to the onset of RA (9, 10), and recent evidence suggests that one of the pathological triggers of RA is the propagation of antigenic epitopes in RA target tissues that are recognised by ACPAs (11-13). It is, therefore, conceivable that citrullinated autoantigens, which are recognised by specific ACPAs, elicit an immune response in susceptible patients and thereby contribute to the chronic inflammatory state typical of RA.

Diagnostic tests currently in use are based on the detection of disease-specific autoantibodies including RF and ACPAs. The commercial second- and third-generation cyclic citrullinated peptide (CCP2 and CCP3) assays are sensitive and reliable for detecting the presence of ACPAs and on a population-basis, high titres are associated with increased levels of joint erosion (8, 14). These assays utilise a series of cyclised synthetic peptides (CCPs) that are used as surrogate antigens to detect the presence of disease-specific ACPAs even during the early stages of the disease (15). However, anti-CCP2 antibody levels are comprehensively calculated based on coupling of ACPAs to multiple cyclic citrullinated peptides. Thus, it is difficult to determine to which antigen level a specific ACPA had contributed or with which clinical symptoms an individual ACPA may be associated.

Several multi-array techniques have been developed that allow for the simultaneous analysis of different antigens that are recognised by ACPAs, as well as the presence of different ACPA isotypes; it is hoped that these techniques may enable researchers to identify links between ACPA reactivity and RA prognosis (16-20). However, quantitative performance or assay reproducibility has not been well characterised to date. Thus, in order to investigate ACPAs that are specific for unique citrullinated peptides (cit-peptides), we optimised and validated a MagPlex™ bead array system (11) using 13 cit-peptides that were previously reported as epitopes recognised by ACPAs in RA patients. By combining clustering and competitive inhibition experiments, this study aimed to identify anti-cit peptides that may play an important role in the onset and progression of RA.

Materials and methods

This was a validation study for the MagPlex™ bead array system, for which both clustering and competitive inhibition experiments were combined. The serum samples used in the present study were sourced from a network of anonymised human donors (BioIVT, Baltimore, MD, USA) and, as such, ethical approval from an institutional review board was not mandatory, in accordance with the Ethical Guidelines for Medical and Health Research Involving Human Subjects set forth by the Ministry of Health, Labour and Welfare in Japan.

Antigenic peptide synthesis

Cit-peptides that were previously reported as epitopes that were recognised by ACPAs in RA patients (11, 12, 21-33) were synthesised using the F-moc solid-phase peptide synthesis method at SCRUM Inc. (Tokyo, Japan). After the addition of biotin, cit-peptides were cyclised by iodine oxidation and purified to ≥80% using high-performance liquid chromatography with a SunFire C18 Column (100Å, 5 μm, 4.6 mm × 150 mm; Waters Corp., Milford, MA, USA).

Donor serum samples

A total of 51 serum samples of RA patients were purchased from BioIVT

with the intent of achieving a composition that approximates the actual epidemiology of RA. The criterion used for each diagnosis of RA was not recorded. In addition, serum from 50 normal donors (NDs) without a history of human immunodeficiency virus, hepatitis B, hepatitis C, or RA (defined as ND serum) were also acquired from BioIVT. Each serum sample was stored in 0.1 mL aliquots at -80°C until use.

The ACPA level of each serum sample was predetermined using the CCP2 ELISA assay at SRL Inc. (Tokyo, Japan). A CCP2 level of <4.5 U/mL was judged as anti-CCP2 negative (34, 35). RA serum samples with a CCP2 level greater than the median (RA patient No. 26, 36, 43, 44, 46, 47, 49, 50 and 51) were pooled, and this was defined as the sero-positive collected serum (SPCS) sample. ND serum samples that were anti-CCP2 negative (ND patient No. 2, 6, 11, 12, 14, 16, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 36, 38, 39, 43, 44, 46 and 47) were also pooled, and this was defined as the sero-negative collected serum (SNCS) sample.

Luminex MagPlex™ coupling assay: preparation of cit-peptide conjugated fluorescent beads

Synthesised biotinylated cit-peptides were fixed to xMap MagPlex™ microsphere beads with unique spectral identities (Luminex Corp., Austin, TX, USA). The beads (3×10^6) were initially suspended in 50 µL of 0.1 M 2-morpholinoethanesulfonic acid in monohydrate buffer pH 7.0 (MES buffer) (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), 150 µL of 10 mg/mL N-hydroxysulfosuccinimide (Thermo Fisher Scientific Inc., Yokohama, Japan), and 150 µL of 10 mg/mL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Thermo Fisher Scientific Inc.). Once suspended, the beads were then incubated at room temperature (RT) for 20 minutes without light. The supernatant was removed and the beads were washed with 500 µL of 0.1 M MES buffer pH 7.0 twice prior to resuspension in 300 µL of 0.05 mg/mL NeutrAvidin solution (Thermo Fisher Scientific Inc.) followed by incubation at RT for 1 hour without light. The NeutrAvidin solution was removed and after washing the avidin-coupled beads (2.5 µg of avidin per 0.5×10^6 beads) twice with 500 µL of phosphate-buffered saline (PBS), the beads were then suspended in PBS for storage.

The biotinylated cit-peptide solution was adjusted to a predetermined concentration (5 µg/mL: Clu 221, Clu 231, Fib 211, Fib 41, Fib 556, Fib 616, Fil 48, H2A 1, and H2B 62; 10 µg/mL: Apo 277, Big 247, Eno 5, and Vim 58) in PBS, and 300 uL was added to set amounts of avidin-coupled beads; this mixture was then incubated at RT for 30 minutes without light. The cit-peptide coupled beads were washed three times in 500 µL of PBS with 0.05% Tween 20 (PBST) and then suspended in PBST with 1.0% bovine serum albumin (BSA) at a concentration of 1×10^6 beads/mL prior to storage at -80°C under light-blocking conditions.

ACPA assay: measurement of cit-peptides in serum samples from RA patients and NDs

Beads that were coupled with each cit-peptide were mixed in equal amounts, and then seeded at 2×10^3 beads/well in a 96-well filter plate (EMD Millipore Corporation, Burlington, MA, USA). Serum samples were diluted (range: 1:50 to 1:819,200) with 1.0% BSA and 4% (w/v) BlockAce (DS Pharma, Osaka, Japan) in PBST, and 100 µL/well of each serum dilution was added to each plate. Plates were gently shaken at RT for 1 hour and the beads were washed three times with PBST. Next, 100 µL of phycoerythrin (PE) labelled goat-poly anti-human-IgG-Fc (PE-anti-hIgG; Bethyl Laboratories, Inc., Montgomery, TX, USA) at a dilution of 1:200 was added, and incubated for 1 hour at RT. After 1 hour, the beads were washed, and the fluorescence intensity was measured using the Luminex® System (Luminex 100 or Luminex 200: Luminex Corp.). Each value was illustrated as a median fluorescence intensity (MFI).

Subtracted MFI (sMFI) was defined as the MFI obtained from each peptide bead minus the MFI of beads without a coupled peptide. The mean and standard deviation values of the sMFI obtained from ND serum samples were calculated for each peptide, and Z-scores for ND and RA patient samples were calculated. The serum sample was deemed to have coupled with the beads if the Z-score exceeded 2.

Inhibition with free cit-peptides: specificity confirmation by inhibition test with free cit-peptides

In the inhibition test, serum samples were diluted 1:100 with 1.0% BSA and 4% (w/v) BlockAce in PBST and incubated with free cit-peptides to a final concentration of 25 µg/mL at RT for 1 hour. These samples were then added to the plate (100 µL/well) and mixed with pre-fixed cit-peptides at RT for 1 hour.

Statistical analysis

The Wilcoxon test was used for comparisons of the distribution of sMFI values. For clustering, the amap package and heatmap.2 function were used. The Pearson method was used to calculate the distance of the binary vector, and the word method was used for clustering. The linear regression analysis was performed using the lm function. The Round Robin *t*-test was used to compare the distribution of inhibition rates for each peptide. The Holm method was used to adjust the multiple test. All statistical analyses were performed using the R language.

Results

Peptide and serum characteristics

The sequence and purity of each synthesised cit-peptide are listed in Table I. The purity of each cit-peptide was >80% and the molecular weights ranged between 2175.4 and 2653.0 g/mol.

The summary of characteristics of each serum sample is shown in Table II. Samples were purchased with the intent of achieving a composition that approximates the actual epidemiology of RA. Among the RA patient samples obtained, the proportion of women was 74.5% and the mean age was 61.1 years, similar to the epidemiology of RA. All 50 ND serum samples were anti-CCP2 negative, while 90.2% (46/51) of RA serum samples were anti-CCP2 positive.

Table I. Characteristics of synthesised peptides.

Original protein	Abbreviation	Modification	Amino acid sequence	Purity (%)	Peptide MW	Reference
Apolipoprotein E 277-296	Apo 277	2 citrullines, cyclic, biotinylated	Biotin-Ahx-A[Cit]LKSWFECPLVEDMQ [Cit]QWAGC-NH2	100	2653	(9, 19, 20)
Biglycan 247-266	Big 247	3 citrullines, cyclic, biotinylated	Biotin-Ahx-CEDLL[Cit]YSKLY [Cit]LGCGHNQI[Cit]-NH2	95.03	2537.9	(9, 20)
Clusterin 221-240	Clu 221	1 citrulline, cyclic, biotinylated	Biotin-Ahx-CQTHMLDVMQDHFS [Cit]ASSIIDC-NH2	100	2535.9	(9)
Clusterin 231-250	Clu 231	3 citrullines, cyclic, biotinylated	Biotin-Ahx-CHFS[Cit]ASSCIDELFQD [Cit]FFT[Cit]-NH2	93.94	2565.8	(9)
Enolase 1A 5-21	Eno 5	2 citrullines, cyclic, biotinylated	Biotin-CKIHA[Cit]EIFDS [Cit]GNPTVEC	90.96	2175.4	(9, 21, 22, 23)
Fibrinogen A 211-230	Fib 211	2 citrullines, cyclic, biotinylated	Biotin-Ahx-CDLLPS[Cit]D[Cit] QHLPCIKMKPVP-NH2	87.13	2445.9	(9, 10, 22, 24, 25, 26)
Fibrinogen A 41-60	Fib 41	3 citrullines, cyclic, biotinylated	Biotin-Ahx-CGGGV[Cit]GP[Cit]VVE[Cit] HQSACKDS-NH2	99.3	2198.4	(9, 10, 22, 24, 25, 26)
Fibrinogen A 556-575	Fib 556	1 citrulline, cyclic, biotinylated	Biotin-Ahx-NTKESSSHHPGCAEFPS[Cit] GKC-NH2	98.53	2257.4	(9, 10, 22, 24, 25, 26)
Fibrinogen A 616-635	Fib 616	3 citrullines, cyclic, biotinylated	Biotin-Ahx-CTHSTK[Cit]GHAKS[Cit] PV[Cit]GIHTSC-NH2	89.82	2419.7	(10, 20, 22, 24, 25, 26)
Filaggrin 48-65	Fil 48	2 citrullines, cyclic, biotinylated	Biotin-Ahx-CTIHAHPGS[Cit][Cit] GGRHGYHHC-NH2	95.78	2238.4	(9, 24, 27)
H2A/a 1-20	H2A 1	3 citrullines, cyclic, biotinylated	Biotin-Ahx-MSG[Cit]GKQGCKA[Cit] AKAKT[Cit]SSC-NH2	92.92	2211.6	(9, 28)
H2B/a 62-81	H2B 62	2 citrullines, cyclic, biotinylated	Biotin-Ahx-CIMNSFVNDIFE[Cit] IAGEAS[Cit]LC-NH2	80.56	2487.8	(9, 28, 29)
Vimentin 58-77	Vim 58	3 citrullines, cyclic, biotinylated	Biotin-Ahx-CGGVYAT[Cit]SSAV[Cit] L[Cit]SSVPGVC-NH2	94.43	2225.5	(9, 20, 30, 31)

Ahx: 6-aminohexanoic acid; cit: citrulline; MW: molecular weight.

Linearity, reproducibility, and sample stability validation

First, the constructed system was evaluated. High linearity (R^2 >0.98) was found for a sample dilution series and MFI values, confirming that this assay was quantitative with respect to ACPAs that can couple with beads (Supplementary Fig. S1).

Differing inter-day and inter-well MFIs were evaluated using SPCS and SNCS to confirm the reproducibility of the system. In all experiments, the percent relative standard deviation (%RSD) was 0.7% to 11.8% (Suppl. Table S1), suggesting that the reproducibility of the system was high.

Assay materials were stored at -80°C and then thawed once on days 1, 50, 80, 125, and 255, prior to use in the ACPA assay (Suppl. Fig. S2). For each fixed cit-peptide, a similar MFI value was obtained across each thaw date and %RSD had a distribution of within 7% (data not shown). This suggests that the assay materials are stable at -80°C for up to 8 months.

Table II. Characteristics of serum samples from rheumatoid arthritis patients and normal donors.

	ND serum samples n=50	RA serum samples n=51
Sex, female, n (%)	25 (50.0)	38 (74.5)
Age, years, mean ± SD	42.3 ± 13.0	61.1 ± 11.6
CCP2 positivity, n (%)	0 (0.0)	46 (90.2)
CCP2 titre, mean ± SD	-	107 ± 126

CCP2: second-generation cyclic citrullinated peptide; ND: normal donor; RA: rheumatoid arthritis; SD: standard deviation.

Binary profile of positivity of cit-peptides and the relationship between the total number of positive peptides and anti-CCP2 antibody titre in ND and RA serum samples

The distribution of sMFI values from all ND and RA serum samples is summarised in Figure 1. Analysis by Wilcoxon test showed that sMFI values were significantly higher in RA serum samples compared with ND serum samples for all citrullinated peptides. For each citrullinated peptide, the binary value (positivity: Z-score ≥2, negativity: Z-score <2) was calculated as an indicator of the presence or absence of coupling, the number of positive peptides in RA

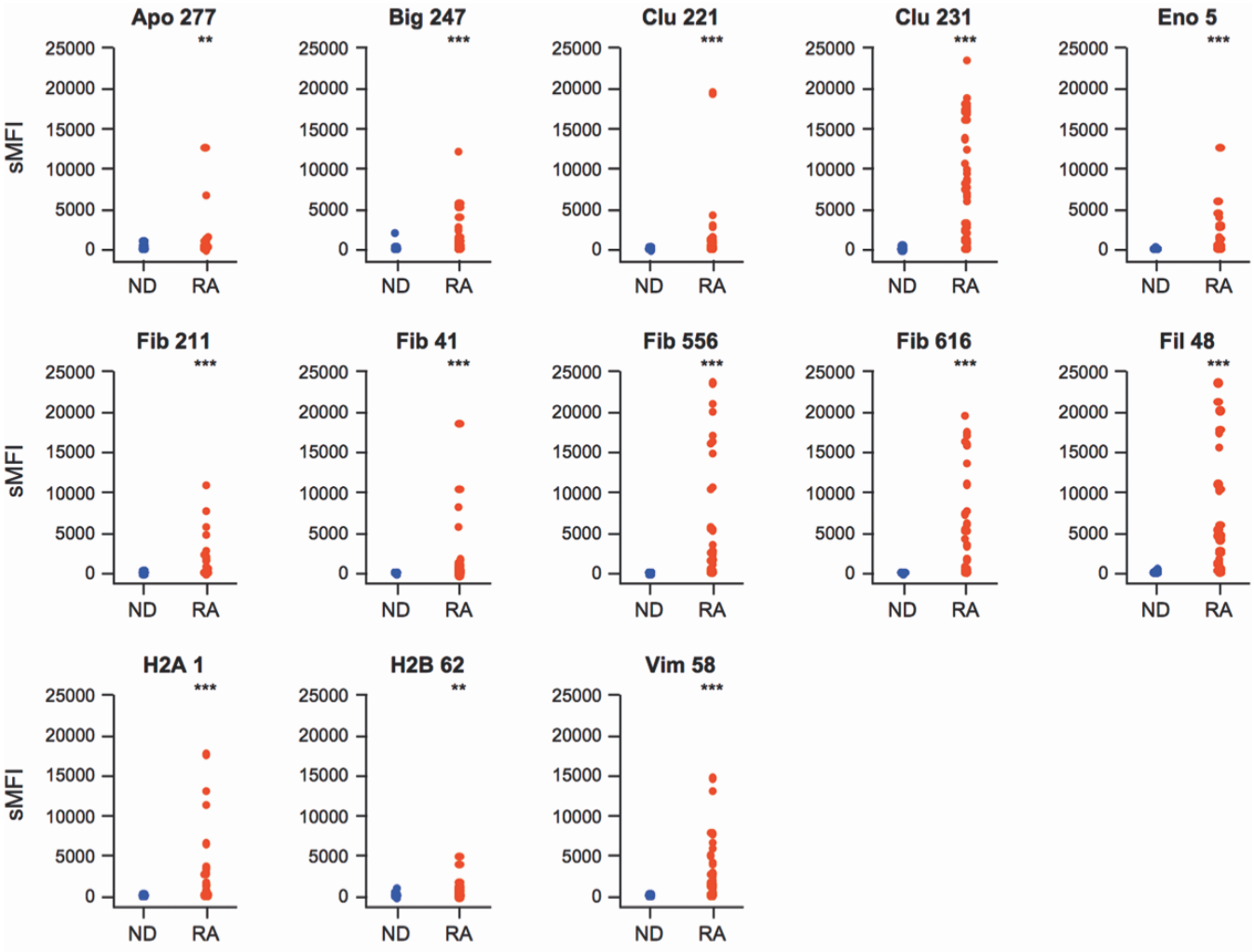

Fig. 1. Distribution of sMFI values from ND or RA serum samples with each fixed cit-peptide.
Diluted (1:100) ND or RA serum samples were incubated with fixed cit-peptides at room temperature for 1 hour, and then probed with PE-anti-hIgG. Fluorescence intensity was measured with the Luminex® System: Distribution of MFI in reactions of ND or RA serum samples with each fixed cit-peptide is shown. The Wilcoxon test was used for comparison of the distribution of sMFI values from ND and RA serum samples.
$^{**}p<0.01$, $^{***}p<0.001$
cit-peptide: citrullinated peptide; ND: normal donor; PE-anti-hIgG: phycoerythrin labelled goat-poly anti-human-IgG-Fc; RA: rheumatoid arthritis; sMFI: subtracted median fluorescence intensity.

serum samples and ND serum samples was determined, and the binary value was visualised by means of a heat map (Fig. 2A). The number of positive peptides was greater in RA serum samples compared with ND serum samples for all citrullinated peptides. Sporadic positivity was also found in ND serum samples. There were seven peptides for which over half of the RA patient samples were positive: Clu 221, Clu 231, Fib 556, Fib 616, Fil 48, H2A 1, and Vim 58. The number of positive samples was lowest for Apo 277, at nine.
When the log of the number of positive peptides and anti-CCP2 antibody titre were plotted for each serum sample, we observed a correlation indicating that the number of positive peptides increased as the anti-CCP2 antibody titre increased (Fig. 2B). The coefficient of determination in regression analysis was relatively high at R^2=0.86.

Binary profile of positivity of cit-peptides in RA patients

Clustering analysis was performed using the binary profile of each peptide for each RA patient sample (Fig. 3). With respect to clustering between citrullinated peptides, an orange cluster comprised of the five peptides Fib 556, Fib 616, Fil 48, Clu 231, and Vim 58, and a yellow cluster consisting of the four peptides Fbi 211, Eno 5, Clu 221, and H2A 1, were formed. The peptides in the orange cluster were positive in most RA patients.
With respect to clustering between patients, three clusters were formed as follows: a blue cluster in which all peptides were negative, a purple cluster in which peptides in the orange and yellow clusters were relatively positive, and a red cluster in which the orange cluster was positive but the peptides in the yellow cluster tended to be negative. Anti-CCP2 antibodies were negative in the RA patient serum in the blue cluster.

Variety of ACPA compositions revealed by the inhibition test with free cit-peptides

Five samples (26, 43, 44, 46, and 51)

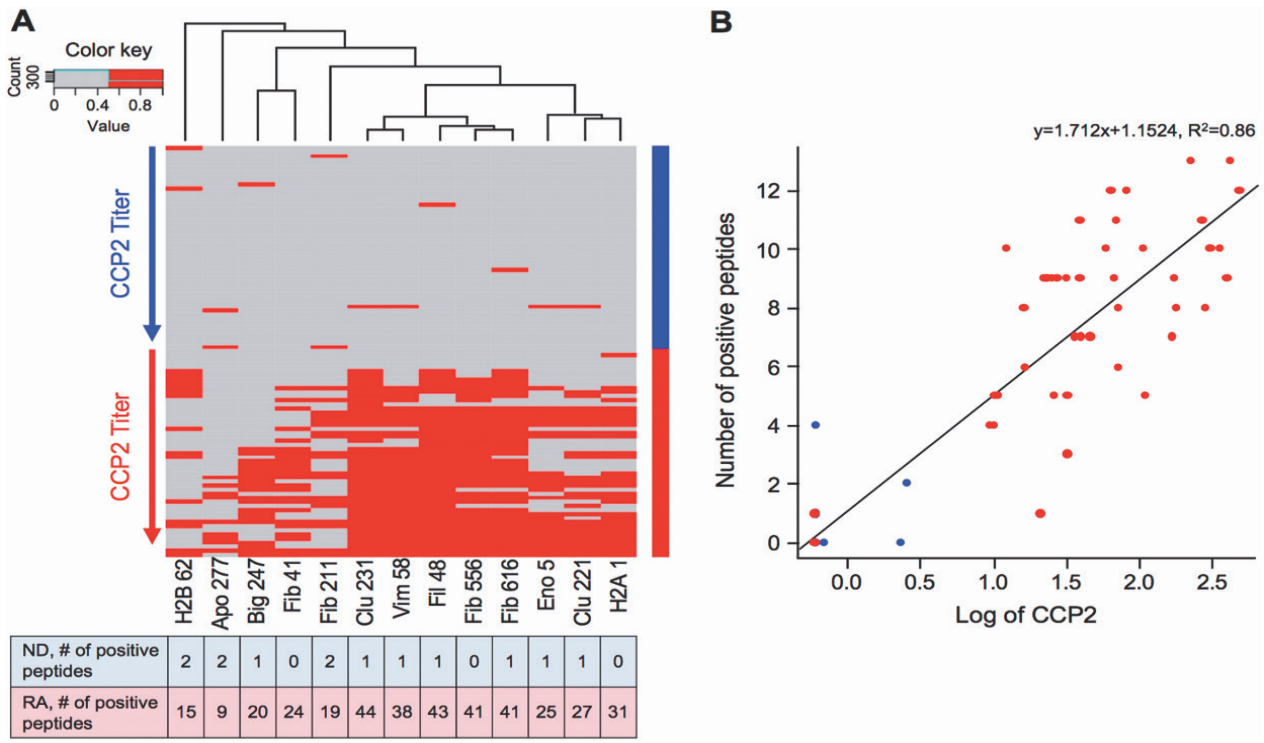

Fig. 2. Association between positive peptide profile and number of positive peptides and CCP2 titre.
A: Positive peptide profile. Blue indicates ND and red signifies RA on the bar on the right. The respective serum samples are presented in order of anti-CCP2 antibody titre. Red indicates positive and grey represents negative in the heat map. For distance between peptides, the Ward method was used as a binary clustering method.
B: Association between the number of positive peptides and CCP2 titre by serum sample. Blue indicates ND and red signifies RA.
CCP2: second-generation cyclic citrullinated peptide; ND: normal donor; RA: rheumatoid arthritis.

were randomly selected from among the RA serum samples, in which the number of positive peptides was ≥10, and were used in the analysis. The inhibition rate was calculated with sMFI without addition of an inhibitory peptide regarded as 0, and with addition regarded as 1. The mean inhibition rate of five samples is shown as a heat map for the combination of beads and inhibitory peptides (Fig. 4A). The mean inhibition rate of other peptides relative to the inhibitory peptides is shown in a graph (Fig. 4B). Analysis of variance showed that the distribution of each peptide, as shown in Figure 4B, was not the same (p<0.001). Round-robin t-testing of each peptide showed that the distribution of Clu 231, Vim 58, and Eno 5 differed from that of other peptides, suggesting that Clu 231, Vim 58, and Eno 5 may affect the MFI from other peptide beads (*i.e.* they may inhibit coupling with various ACPAs).

Discussion

In this study, we optimised the custom MagPlex™ bead array (11) and validated its diagnostic performance and reproducibility when evaluating the presence of specific ACPAs in RA serum samples. This optimised assay showed good linearity when used across a series of diluted ACPA-positive RA serum samples with an MFI range up to 10,000.

The sMFIs from the cit-peptides used in this array system differed significantly in NDs and RA patients. The sum of cit-peptides with high intensity in RA patients compared with NDs (*i.e.* the number of positive peptides) roughly correlated with anti-CCP antibody titre. When sensitivity and specificity for distinguishing NDs and RA patients were investigated, the two indicators were comparable with anti-CCP antibody titre (data not shown). Based on the above findings, it was summarised that the number of positive peptides, which can be calculated with this system, was as useful as anti-CCP2 antibodies, at least for the diagnosis of RA patients.

Cluster analysis of patients and peptides based on the assessment of positivity/negativity to each cit-peptide showed that at least two patient groups could be identified, with the exception of patients in whom all tests were negative. Peptides for which the frequency of positivity was relatively high in both groups were Fib 556, Fib 616, Fil 48, Clu 231, and Vim 58. In other words, citrullination of these five proteins at a specific location might have an important effect on the onset and progression of RA.

For H2B 62, Apo 177, Big 247, and Fib 41, the positivity frequency was relatively low, and the positivity pattern was different depending on the individual. It has been reported that somatic mutation of B cells causes change to antibody glycosylation, resulting in ACPAs with a low avidity that are associated with formation of bone erosion in RA (36). This suggests the possible progression of an epitope spreading process that differs from person to person. Our system may be useful for patient stratification and personalised therapy, as the presence of ACPAs that differ from person to person can be confirmed.

This was our first attempt at using this system to perform inhibition experi-

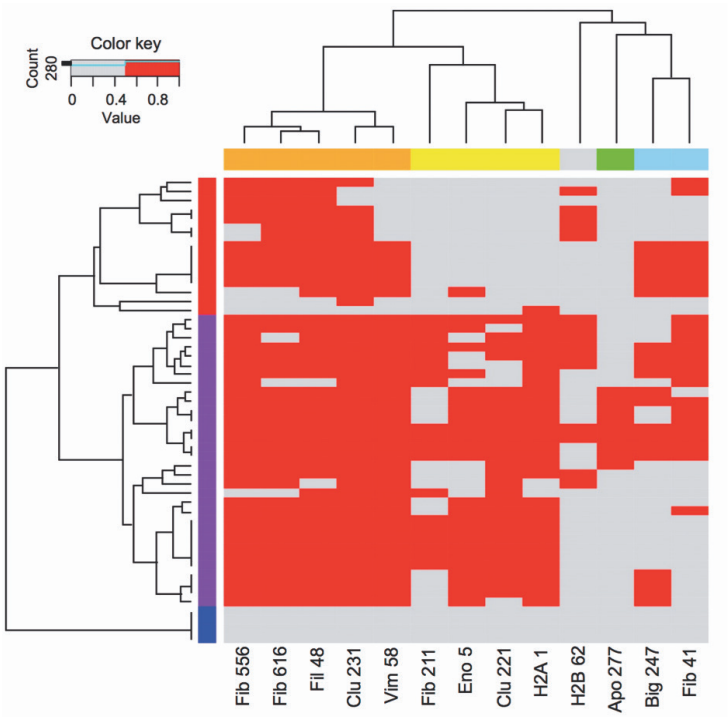

Fig. 3. Positive peptide profile in rheumatoid arthritis patients.
Red indicates positive and grey represents negative in the heat map. For distance between samples and between peptides, the Ward method was used as a binary clustering method.

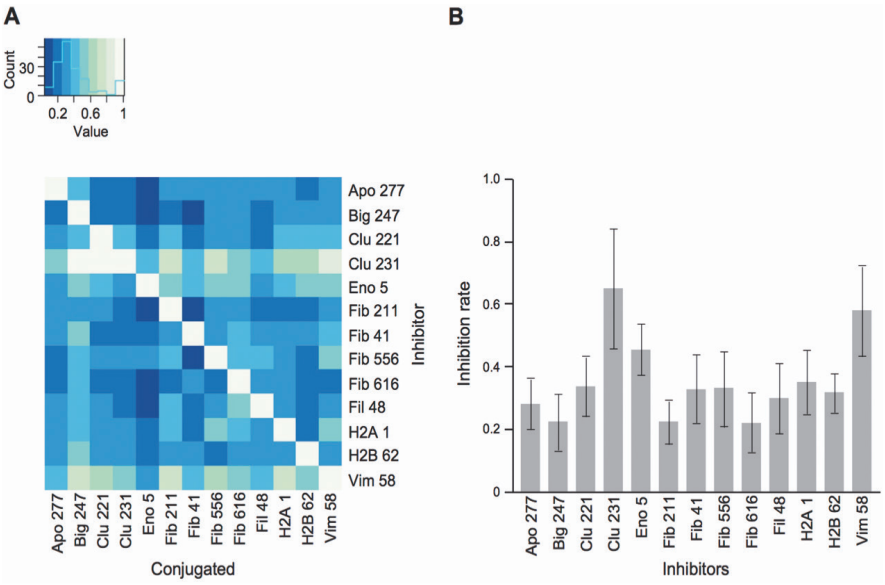

Fig. 4. Reduction of MFI values for each RA serum sample by pre-incubation with free cit-peptide.
RA serum samples (RA 26, 43, 44, 46, and 51) were individually pre-incubated with each free cit-peptide (25 μg/mL at room temperature for 1 hour), and then incubated with each antigen-coated bead set. Fluorescence intensity was measured with the Luminex® System and data are presented as MFI.
A:Heat map showing the combination of beads and inhibitory peptides.
B:Mean inhibition rate of other peptides relative to the inhibitory peptides. 1.0 (y-axis) represents 100% inhibition when the peptides bound to the bead and the inhibitory peptides are the same. Analysis of variance $p<0.001$.
CCP2: second-generation cyclic citrullinated peptide 2; cit-peptide: citrullinated peptide; MFI: median fluorescence intensity; RA: rheumatoid arthritis.

ments using free cit-peptides. It was found that free Clu 231, Eno 5, and Vim 58 may affect the coupling of other cit-peptides with ACPAs. Taken together with the results of cluster analysis, ACPAs that recognise Clu 231 and Vim 58 may be the most primitive component of the pathogenesis of rheumatism, and ACPAs that recognise other cit-proteins may have subsequently occurred due to epitope spreading.

Sokolove and colleagues, the originators of this system (11), demonstrated the relative abundance of ACPAs that recognise Clu 231 and Vim 58 in the blood of patients prior to being diagnosed with RA. This also suggests that these peptides illicit an autoimmune response accompanied by ACPA production as an autoantigen prior to the onset of rheumatism.

Vimentin is an intermediate filament found in various mesenchymal cells such as osteocytes and chondrocytes. Citrullinated vimentin antibodies have been found as anti-Sa in RA patients in the past, and it has been reported that they are more useful than CCP2 antibodies for early diagnosis of RA according to measurements using anti-MCV (37). In addition, citrullination of vimentin reportedly alters the vimentin filament network, causing condensing around the nucleus, which triggers apoptosis (38). It has been reported that citrullinated vimentin is detected in monocytes and macrophages treated with ionomycin (39). Given the above findings, vimentin citrullination may be triggered via apoptosis of macrophages activated for a long period of time locally in inflamed joints, and it may play an

important role in the onset of RA (37). Clusterin is a ubiquitous, extracellular chaperon that prevents aggregation of stress-induced proteins (40). It has been reported that decreased expression of clusterin is seen in synovial fibroblasts in RA, and the decrease at the intracellular level promotes the degradation of nuclear factor kappa B (NF-κB) inhibitor alpha (IκBα), which affects NF-κB signalling and may promote expression of interleukin-6 and interleukin-8 (41). It also reportedly binds with matrix metallopeptidase 9 precursors and activators involved in articular destruction and inhibits their enzyme activity (42). Thus, clusterin is thought to be closely associated with the onset and progression of RA.

We were unable to find any previous reports in the literature that focused on the relationship between citrullinated clusterin and RA, and its role in the pathogenesis of RA remains unclear. However, a localised clusterin abnormality may occur as a result of abnormal regulation of expression, leading to progression of a citrullination response that would not normally occur and to ACPA production.

The present study has some limitations. The assay was conducted with a limited number of serum samples of both RA patients and healthy donors. As the serum samples used in this study were purchased, we were not able to verify the relationship between patient background characteristics and RA disease activity as in actual clinical practice. However, despite these limitations, in the future, this assay system may be used in clinical studies to clarify the role of ACPA specificity in the pathogenesis of RA.

In summary, we developed a custom ACPA assay incorporating 13 RA-specific cit-peptides and used this assay to investigate ACPAs in serum samples from RA patients. Our assay exhibited good sensitivity and reproducibility across a range of RA serum samples. The results of evaluation of the number of positive peptides using Z-score and inhibition experiments using free cit-peptides suggested the importance of ACPAs that recognise specific citrullinated clusterin and vimentin. This assay may provide an effective diagnostic tool to assess how differing ACPA compositions may play a role in different RA subtypes. Further investigation into the relationship between different ACPAs and disease symptoms using this optimised assay may expand our understanding of the role ACPAs play in joint erosion and a patient's quality of life.

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