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Original article

Anti-citrullinated peptide antibody-negative RA is a genetically distinct subset: a definitive study using only bone-erosive ACPA-negative rheumatoid arthritis

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Abstract

Objectives. ACPA is a highly specific marker for RA. It was recently reported that ACPA can be used to classify RA into two disease subsets, ACPA-positive and ACPA-negative RA. ACPA-positive RA was found to be associated with the HLA-DR shared epitope (SE), but ACPA negative was not. However, the suspicion remained that this result was caused by the ACPA-negative RA subset containing patients with non-RA diseases. We examined whether this is the case even when possible non-RA ACPA-negative RA patients were excluded by selecting only patients with bone erosion.

Methods. We genotyped HLA-DRB1 alleles for 574 ACPA-positive RA, 185 ACPA-negative RA (including 97 erosive RA) and 1508 healthy donors. We also tested whether HLA-DR SE is associated with RF-negative or ANA-negative RA.

Results. ACPA-negative RA with apparent bone erosion was not associated with SE, supporting the idea that ACPA-negative RA is genetically distinct from ACPA-positive RA. We also tested whether these subsets are based on autoantibody-producing activity. In accordance with the ACPA-negative RA subset, the RF-negative RA subset showed a clearly distinct pattern of association with SE from the RF-positive RA. In contrast, ANA-negative as well as ANA-positive RA was similarly associated with SE, suggesting that the subsets distinguished by ACPA are not based simply on differences in autoantibody production.

Conclusions. ACPA-negative erosive RA is genetically distinct from ACPA-positive RA.

Key words: Rheumatoid arthritis, Anti-citrullinated peptide antibody, HLA, Shared epitope, Subset, Genetics, Association study.

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Introduction

RA is an inflammatory arthritic disorder that is characterized by inflammatory cell infiltration, synovial cell proliferation and destruction of cartilage and subcartilageous bones, which can lead to joint deformity. However, the clinical course of RA varies from patient to patient, as do autoantibody profiles such as RF and ACPA. Such heterogeneity may be derived from genetic and environmental factors. In the early stage of arthritis, the diagnosis of RA is often difficult and such patients can be classified as

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undifferentiated arthritis (UA). According to Thabet *et al*. [1], about half of the UA patients remit spontaneously, while ~30% develop RA. At baseline, 28.6% of UA patients have bone erosions and it is a good prognostic value for the development of RA.

ACPA is an autoantibody that recognizes peptides or proteins whose arginine residues are changed to citrulline by post-translational modification. The target protein is not a single protein, but filaggrin [2], vimentin [3], fibrin [4], α-enolase [5] and so on. ACPA is a useful diagnostic marker for RA because of its very high specificity (>95%) and reasonably high sensitivity (65–88%) [6–8]. It has also been proposed that ACPA is a useful marker for predicting destructive RA [9, 10].

Genetic predisposition to RA has been investigated intensively. HLA is a major determinant of RA susceptibility and *HLA-DRB1 *0101, *0102, *0401, *0404, *0405, *0408, *0410, *1001, *1303* and **1402* are reported to be associated with RA development. There is a common amino acid sequence among such HLA-DR molecules at the 70th–74th residues of the HLA-DRβ1 chain, which is called a shared epitope (SE) [11]. The association of carrying this SE and developing RA has been repeatedly reported for different ethnic groups. However, recently, a Dutch group reported that the association of SE was only exhibited with ACPA-positive RA and no association was seen with the ACPA-negative RA patients [12]. They also showed that the influence of SE on joint damage was abrogated when stratified by ACPA. In addition to *HLA-DRB1* (SE), other RA susceptibility genes such as *PTPN22, CTLA4, TRAF1/C5* and *STAT4* were also investigated for association by stratifying RA with ACPA [13–16]. In almost all cases, such susceptibility genes were found to be associated with ACPA-positive RA but not with ACPA-negative RA. Although genetic differences are clear between ACPA-positive and ACPA-negative RA, there still remains the possibility that such differences might be caused by the contamination of non-RA diseases such as seronegative SpA and PMR in the ACPA-negative RA subset. In this article, we re-evaluated the association analysis by selecting only patients with bone-eroding arthritis for the ACPA-negative population.

Materials and methods

Patients and healthy control subjects

A total of 1411 patients who were diagnosed with RA in five hospitals (Kyoto University Hospital, Dohgo Spa Hospital, Sagamihara National Hospital, Niigata Rheumatic Center and Saiseikai Takaoka Hospital) were enrolled in this study. All patients were Japanese and fulfilled the ACR (formerly ARA) 1987 revised criteria for the classification of RA. RA patients overlapped with other collagen vascular diseases were excluded. SS was not excluded because the prevalence of SS in our cohort was quite low (<2%) compared with the reported prevalence of 10–24%, probably due to incomplete clinical information. The ethics committee of each hospital approved the study and genomic DNA was extracted from peripheral blood of patients and healthy individuals after written informed consent was obtained. Out of 1411 RA patients, 1182 (83.8%) were ACPA positive and 229 (16.2%) were ACPA negative. Five hundred and seventy-four ACPA-positive and 185 ACPA-negative RA patients were selected and genotyped for *HLA-DRB1*. Out of the 185 ACPA-negative RA patients, radiographic data were available in 160 patients, of whom 97 patients had typical bone erosions. Such patients are denoted as ACPA-negative erosive RA patients in this article. DNA samples from 1508 healthy control subjects were collected at Aichi Cancer Center Hospital and from the DNA banks for healthy Japanese volunteers of the Pharma SNP Consortium [17] after written informed consent was obtained.

Genotyping and autoantibody detection

HLA-DRB1 genotyping was carried out with a high-throughput, high-resolution genotyping method (WAKFlow WAKUNAGA) by combining PCR and sequence-specific oligonucleotide probe protocols with the Luminex 100 xMAP flow cytometry dual-laser system to quantify fluorescently labelled oligonucleotides attached to colour-coded microbeads. The following *HLA-DRB1* alleles were classified as SE positive: *DRB1*0101, *0102, *0401, *0404, *0405, *0408, *0413, *0416, *1001, *1303* and **1402*.

ACPA in sera or plasma was detected using a second-generation anti-CCP antibody (Ab) ELISA kit (MESACUP CCP; Medical & Biological Laboratories Co. Ltd, Nagoya, Japan) in accordance with the manufacturer's instructions. A cut-off value of 4.5 U/ml was used for anti-CCP Ab positivity. RF was quantified by latex immunoturbidimetry and the cut-off values of each detection kit in each hospital were employed. ANA was semi-quantified by IIF for most samples, but some were measured by ELISA (MESACUP ANA; Medical & Biological Laboratories Co. Ltd). The cut-off values of each hospital were employed.

Statistical analysis

Chi-squared test, Student's *t*-test, Jonckheere–Terpstra trend test and the 95% CI of odds ratio (OR) were used to assess the statistical significance and magnitude of association for categorical outcomes.

Results

ACPA-positive RA is distinct from ACPA-negative RA on the basis of SE association

One hundred and eighty-five ACPA-negative patients and 574 ACPA-positive patients, as well as 1508 healthy individuals, were genotyped for *HLA DRB1*. SE was determined as described in the 'Materials and methods' section. ACPA was not tested for healthy individuals because its positivity among healthy people was reported to be only ~1% [6, 18]. As shown in Tables 1 and 2, SE was the clear risk factor for ACPA-positive RA development ($P = 8.7 \times 10^{-32}$ and 5.3×10^{-28} for double- and

Table 1 Association of SE with ACPA-positive or ACPA-negative RA

SE status	Control ($n = 1508$)	ACPA-positive RA ($n = 574$)		ACPA-negative RA ($n = 185$)		ACPA-negative erosive RA ($n = 97$)	
	n (%)	*n* (%)	OR (95% CI)	*n* (%)	OR (95% CI)	*n* (%)	OR (95% CI)
SE (+/+)	74 (5)	93 (16)	6.6 (4.7, 9.3)	6 (3)	0.7 (0.3, 1.7)	3 (3)	0.7 (0.2, 2.3)
SE (+/−)	492 (33)	302 (53)	3.2 (2.6, 4.0)	71 (38)	1.3 (0.9, 1.7)	39 (40)	1.4 (0.9, 2.1)
SE (−/−)	942 (62)	179 (31)	1.0	108 (58)	1.0	55 (57)	1.0

SE (+/+): double-SE carrier; SE (+/−): single-SE carrier; SE (−/−): no SE carrier.

Table 2 *P*-values for association of SE between each group

Groups for comparison	*P*-value		
	SE (+/+) *vs* SE (−/−)	SE (+/−) *vs* SE (−/−)	SE (+) *vs* SE (−)
Control *vs* ACPA-positive RA	8.7×10^{-32}	5.3×10^{-28}	1.8×10^{-37}
Control *vs* ACPA-negative RA	0.43	0.16	0.28
Control *vs* ACPA-negative erosive RA	0.54	0.16	0.26
ACPA-positive RA *vs* ACPA-negative RA	2.9×10^{-9}	1.0×10^{-7}	3.3×10^{-11}
ACPA-positive RA *vs* ACPA-negative erosive RA	1.0×10^{-5}	1.2×10^{-4}	1.1×10^{-6}
ACPA-negative RA *vs* ACPA-negative erosive RA	0.98	0.77	0.79

P-values were calculated by chi-squared test.

single-SE carriers, respectively), but not for ACPA-negative RA development ($P = 0.43$ and 0.16 for double- and single-SE carriers, respectively). There was also a dose effect of SE number for ACPA-positive RA (ORs were 6.6 and 3.2 for double- and single-SE carriers, respectively), but not for ACPA-negative RA (ORs were 0.71 and 1.3 for double- and single-SE carriers, respectively). When combining the double- and single-SE carriers, *P*-values for ACPA-positive RA *vs* control, ACPA-negative RA *vs* control and ACPA-positive RA *vs* ACPA-negative RA were 1.8×10^{-37}, 0.28 and 3.3×10^{-11}, respectively. These results are similar to those obtained for Caucasian [12] and Japanese subjects [19].

SE was not associated with ACPA-negative RA even when selecting only bone-destructive RA patients

As reported previously [12], no association was observed between SE and ACPA-negative RA. However, some of the patients who were diagnosed with ACPA-negative RA might be non-RA patients, such as those with seronegative SpA, PMR, palindromic rheumatism, OA and other collagen vascular diseases. Indeed, during a survey of the medical records, we found three patients in the ACPA-negative RA subset who had been diagnosed with MCTD, SLE or PMR and were subsequently recorded as presenting with RA. Although we cannot tell which diagnosis is correct, such cases led us to the idea that it is important to exclude possible non-RA patients in ACPA-negative RA subset in order to reveal whether SE is really not associated with ACPA-negative RA. We first excluded the patients who had suffered from RA for <3 years in order to exclude patients with potentially false-negative results for ACPA, on the basis of the fact that the sensitivity of ACPA is lower in the early stage of RA than in the established stage of RA (disease duration $\geqslant 3$ years) [7]. Then, we excluded possible non-RA patients who do not have bone erosions by X-ray. Ninety-seven ACPA-negative RA patients showed typical bone erosions and were denoted as ACPA-negative erosive RA patients. As shown in Table 3, the baseline characteristics of ACPA-negative erosive RA patients are similar to those of ACPA-positive RA patients. However, an association of SE with ACPA-negative erosive RA was not observed (Tables 1 and 2). The *P*-value for ACPA-negative erosive RA against the control was 0.26; in contrast, that for ACPA-positive RA against the control was 1.8×10^{-37}. This result clearly shows that ACPA-negative erosive RA is a distinct subset from ACPA-positive RA ($P = 1.1 \times 10^{-6}$), and HLA-DRs containing SE are not causative alleles for developing ACPA-negative RA.

RF, but not ANA, positivity classified RA in terms of SE association

Since it was previously reported that SE was associated only with RF-positive RA [20, 21], we also tested this with our cohort. RF data were available for 843 RA patients and 85.6% were positive for RF. As shown in Table 4, SE was significantly associated with RF-positive RA ($P = 1.0 \times 10^{-44}$, OR 3.7), while the association was much weaker with RF-negative RA ($P = 2.2 \times 10^{-4}$, OR 2.0), showing similar results to Caucasian subjects.

We hypothesized that SE may be related to autoantibody production in general, because not only ACPA and RF but also anti-calpastatin antibodies are reported to be

associated with SE [22]. Therefore, we further examined the association between SE and ANA positivity in RA. ANA data were available for 491 RA patients: 385 (78.4%) patients were ANA positive (Table 5). In contrast with ACPA and RF results, SE was equally associated with both ANA-positive and ANA-negative RA ($P = 3.1 \times 10^{-29}$, OR 3.8 and $P = 6.4 \times 10^{-9}$, OR 3.2, respectively), indicating that ANA does not classify RA in terms of SE. Even when the cut-off value of ANA was set higher, the result was similar (data not shown).

Table 3 Baseline characteristics of ACPA-positive RA and ACPA-negative erosive RA

Characteristics	ACPA-positive RA ($n = 574$)	ACPA-negative erosive ($n = 97$)	*P*-value*
Age, mean (S.D.), years	63.0 (12.8)	62.1 (12.6)	0.83
Sex: women, %	81.6	86.0	0.29
Disease duration, mean (S.D.) years	18.3 (11.9)	18.0 (13.9)	0.95
Stage, *n* (%)			
1	52 (9.1)	0 (0)	
2	100 (21.4)	26 (27)	
3	69 (14.8)	25 (26)	
4	246 (52.7)	46 (47)	
Class, mean (S.D.)	1.82 (0.69)	2.07 (0.65)	

As not all of the X-ray films for ACPA-positive RA patients were available, the total number of patients and the sum of patients for stage classification do not match. *Student's *t*-test was used for statistical analysis. The *P*-values for stage and class classification are not shown because non-erosive patients were intentionally excluded from the ACPA-negative subset.

SE (especially *DRB1*0405*) is associated with ACPA titre but not RF nor ANA titre

We also investigated whether SE is related to autoantibody titres. ACPA, RF and ANA titres were measured only for the sera from the Kyoto University cohort. The sera with an ACPA titre >100 IU/ml were further diluted to obtain a correct titre. Among those for whom HLA data were available, 252, 248 and 173 RA patients were positive for ACPA, RF and ANA, respectively. Only samples positive for each autoantibody were selected and the association of each autoantibody titre with SE number was tested by Jonckheere–Terpstra trend test. As shown in Fig. 1A–C, the number of SEs is associated with ACPA titre, but not with RF or ANA titre. When we focused on the *DRB1*0405* allele (the most popular SE allele in Japanese subjects), the association of ACPA titre and *DRB1*0405* allele number was statistically significant ($P = 0.000127$) as shown in Fig. 2.

Discussion

Here, we have demonstrated that *HLA-DRB1* SE is associated with ACPA-positive RA, but not with ACPA-negative RA in Japanese subjects. No association of SE with ACPA-negative RA was observed even when eliminating possible non-RA patients from the ACPA-negative RA group. We further demonstrated that ANA did not classify RA into two subsets in terms of SE association, in contrast with RF and ACPA.

The fact that ACPA-positive and ACPA-negative RA are genetically distinct subsets was first reported by a Dutch group studying Caucasian subjects [12], followed by a group studying Japanese subjects [19]. However, the number of patients enrolled in the Japanese

Table 4 Association of SE with RF-positive or RF-negative RA

SE status	Control ($n = 1508$) *n* (%)	RF-positive RA ($n = 722$)		RF-negative RA ($n = 121$)		*P*-value*
		n (%)	OR (95% CI)	*n* (%)	OR (95% CI)	
SE (+/+)	74 (5)	113 (16)	6.5 (4.7, 9.0)	11 (9)	2.5 (1.3, 5.1)	0.0061
SE (+/−)	492 (33)	387 (54)	3.3 (2.7, 4.1)	55 (45)	1.9 (1.3, 2.8)	0.0072
SE (−/−)	942 (62)	222 (31)	1.0	55 (45)	1.0	

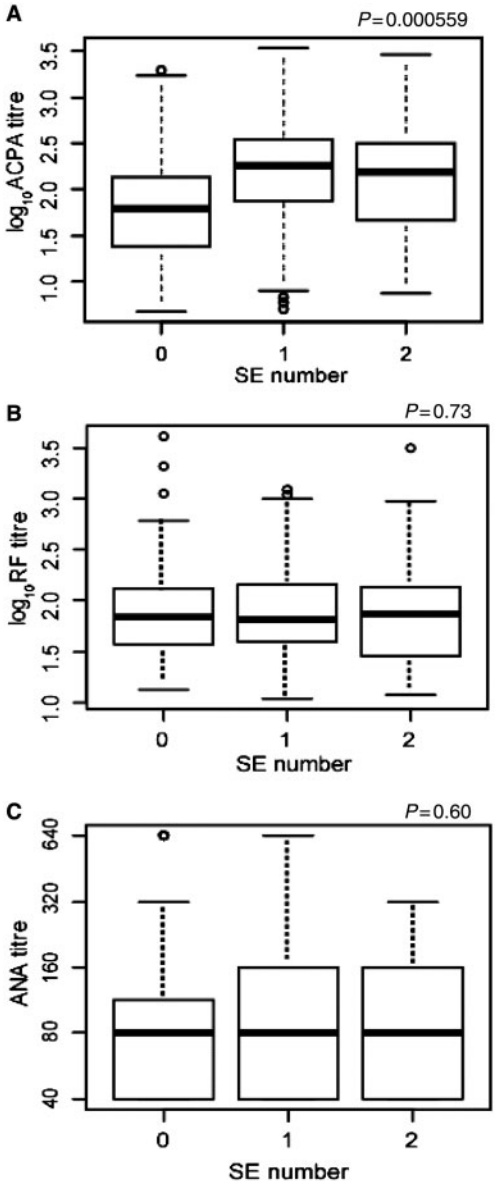
**P*-value for RF-positive *vs* RF-negative RA by chi-squared test.

Table. 5 Association of SE with ANA-positive or ANA-negative RA

SE status	Control ($n = 1508$) *n* (%)	ANA-positive RA ($n = 385$)		ANA-negative RA ($n = 106$)		*P*-value*
		n (%)	OR (95% CI)	*n* (%)	OR (95% CI)	
SE (+/+)	74 (5)	53 (14)	5.7 (3.8, 8.5)	20 (19)	7.1 (3.9, 12.8)	0.51
SE (+/−)	492 (33)	214 (56)	3.5 (2.7, 4.5)	50 (47)	2.7 (1.7, 4.1)	0.28
SE (−/−)	942 (62)	118 (31)	1.0	36 (34)	1.0	

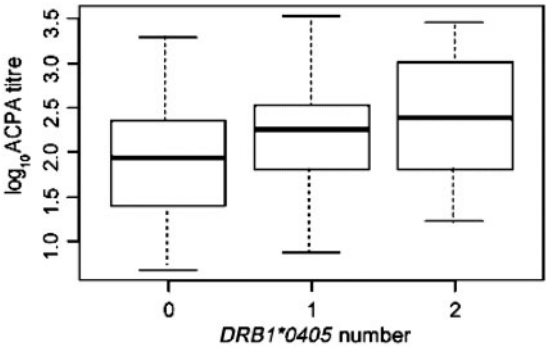
**P*-value for ANA-positive *vs* ANA-negative RA by chi-square test.

Fig. 1 Association of number of SE alleles and titre of ACPA, RF or ANA. ACPA-positive (**A**), RF-positive (**B**) or ANA-positive (**C**) RA patients were selected from the Kyoto University cohort, and the serum ACPA titre (**A**), RF titre (**B**) or ANA titre (**C**) was plotted stratified by the number of SE alleles present. The *P*-values were calculated by Jonckheere–Terpstra trend test.



study was only 110 RA patients (82 ACPA-positive and 28 ACPA-negative) and the *P*-values were 0.017 and 0.033 for double-SE and single-SE carriers, respectively. Furthermore, both the Dutch and the Japanese groups enrolled only early RA patients. Therefore, as we discuss later, their cohorts might have contained non-RA patients, especially in the ACPA-negative group. Since our ACPA-negative RA cohort consisted only of patients with established RA (disease duration >3 years) and the *P*-value

Fig. 2 Association of *HLA-DRB1*0405* allele number and ACPA titre. Only ACPA-positive RA samples were selected from the Kyoto University cohort, and ACPA titres and the number of *HLA-DRB1*0405* alleles (the most popular SE allele in Japanese subjects) in each sample are box plotted. The *P*-value by Jonckheere–Terpstra trend test for this association is 0.000127.



reached 3.3×10^{-11}, our study may be the first that has clearly shown that ACPA-positive and ACPA-negative RA subsets are distinct based on SE association using an established RA cohort.

One of the major issues that we aimed to clarify was the suspicion that the lack of an association of SE with ACPA-negative RA was due to ACPA-negative RA groups including non-RA patients. Since the specificity of ACR (formerly ARA) 1987 revised criteria for the classification of RA has been reported to be 89% [23] and it is probable that many non-RA patients will fall into the ACPA-negative group, it is clear that the ACPA-negative RA patient group contains some non-RA patients, which affects the calculated association. From our survey of medical records, 77 out of 174 ACPA-negative patients for whom records were available did not show any bone erosion by X-ray. These patients might not have RA, although we believe that many of these patients do have RA because the group should include RA patients in remission as well as some with slightly active RA without the exhibition of clear changes detectable by radiography. Since all of the patients in our ACPA-negative erosive RA cohort have bone erosion as determined by X-ray, the number of non-RA patients should be minimal. As shown in Table 3, 73% of ACPA-negative erosive RA patients are classified in Steinbrocker's Stage III or IV with joint deformity. Often ACPA-negative RA is described as a less severe arthritic subset, but our erosive cohort consists of patients with RA of a severity similar to that of ACPA-positive RA. Nonetheless, it is interesting that ACPA-negative RA is genetically distinct from ACPA-positive RA.

The next question we addressed was whether such subsets may be formed generally by autoantibody-producing ability. Since it has already been reported that SE was not associated with RF-negative RA [20, 21] or anti-calpastatin-negative RA [22], it appears that SE is related to autoantibody

production in general. However, ANA did not classify RA into two subsets on the basis of the association with SE. Therefore, SE is related to at least ACPA, RF and anti-calpastatin production, but not ANA, suggesting that HLA-DR molecules with SE consensus amino acid sequence present rather specific autoantigens. The dosage effect of the *DRB1*0405* allele for ACPA titre (Fig. 2), but not RF titre, (data not shown) supports this.

Genetic polymorphisms of *PTPN22, CTLA4, TRAF1/C5* and *STAT4* are also reported to be associated with only ACPA-positive RA but not with ACPA-negative RA [13–16]. There is a circumstantial evidence that smoking may promote citrullination of protein/peptides [24] and the affinity of citrullinated vimentin peptide for SE-containing HLA-DR molecules, *HLA-DRB1*0101, *0401* and *0404*, is higher than that of non-citrullinated vimentin peptide [25]. From these findings, one may assume that SE and other genetic polymorphisms, together with smoking, promote the production of ACPA, resulting in joint inflammation [26]. Although there are no direct evidences that ACPAs cause arthritis, aggravation of experimental arthritis by transferring anti-citrullinated fibrinogen mAbs was demonstrated [27], suggesting an arthritis-promoting activity of ACPA. So, we assume that antigen-presenting cells expressing HLA with SE may preferentially present citrullinated peptides to Th2 cells, which may support ACPA-producing B lymphocytes to differentiate into plasma cells. In contrast, there are no plausible explanations for the pathogenesis of ACPA-negative RA. Unknown autoantibodies under a different genetic background might cause arthritis in ACPA-negative RA, or antibody-independent mechanism might be a major pathogenesis in ACPA-negative RA. *HLA-DRB1*03* and *0901 were reported to be weakly associated with ACPA-negative RA patients in Caucasian [28, 29] and Japanese [19] groups, respectively, and only a few genetic determinants of ACPA-negative RA among non-HLA genes have been reported [30, 31]. So far, no genome-wide association study for ACPA-negative RA has been reported, and genetic and environmental factors of ACPA-negative RA development is to be elucidated.

Rheumatology key messages

- ACPA-negative RA, even of bone-erosive type, is distinct subset from ACPA-positive RA.
- HLA-DRB1 SE is not associated with ACPA-negative RA.
- SE is associated with ACPA titre, but not with RF or ANA titres.

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