

Differences in the Spectrum of Anti–Citruinated Protein Antibody Fine Specificities Between Malaysian and Swedish Patients With Rheumatoid Arthritis

Implications for Disease Pathogenesis

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Objective. Antibodies to the citruinated protein antigens (ACPAs) are important in the diagnosis and pathogenesis of rheumatoid arthritis (RA). However, the prevalence of ACPAs with different fine specificities in different populations is unclear. This study sought to examine the fine specificity of the antibody responses toward citruinated proteins in RA patients from Malaysia, an area where genetic and environmental determinants of RA are different from those in more frequently studied cohorts of Caucasian subjects.

Methods. A multiplex analytic microarray system was used to analyze the occurrence of antibodies to 10

different citruinated peptides (filaggrin [fil307–324], vimentin [Vim2–17, Vim60–75], fibrinogen [Fib α 563–583, Fib α 580–600, Fib β 36–52, Fib β 62–81a, Fib β 62–81b], enolase [Eno5–21], and type II collagen [CitCII355–378]) in serum samples from 4,089 RA patients (1,231 Malaysian and 2,858 Swedish) and 827 healthy control subjects (249 Malaysian and 578 Swedish). The positive reaction threshold for each peptide was set separately for each population based on a specificity of 98%.

Results. Distinct differences in the frequencies of 5 ACPA fine specificities (Vim60–75, Vim2–17, Fib β 62–81b, Eno5–21, and CitCII355–378) were found between the Malaysian and Swedish RA populations, despite a nearly identical percentage of patients in each population who were positive for anti–cyclic citruinated peptide 2

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antibodies. In Malaysian RA patients compared with Swedish RA patients, the frequencies of antibodies to Vim60–75 (54% versus 44%, corrected $P [P_{\text{corr}}] = 1.06 \times 10^{-8}$) and CitCII355–378 (17% versus 13%, $P_{\text{corr}} = 0.02$) were significantly higher, while the frequencies of antibodies to Vim2–17 (25% versus 32%, $P_{\text{corr}} = 1.91 \times 10^{-4}$), Fib β 62–81b (15% versus 30%, $P_{\text{corr}} = 2.47 \times 10^{-22}$), and Eno5–21 (23% versus 50%, $P_{\text{corr}} = 3.64 \times 10^{-57}$) were significantly lower.

Conclusion. Serum ACPA fine specificities differ between RA patients in different populations, although the total proportions of individuals positive for ACPAs are similar. Differing patterns of ACPA fine specificity could be attributed to variations in genetic and/or environmental factors.

There is overwhelming evidence to indicate that rheumatoid arthritis (RA) is not a single disease entity, but rather can be subclassified on the basis of autoantibodies, such as rheumatoid factor (RF) and anti-citrullinated peptide/protein antibodies (ACPAs) (1–7). ACPAs are highly specific and universal diagnostic biomarkers for RA across different populations (8–12). Furthermore, ACPAs can be detected several years before the onset of RA (13).

ACPAs are detected by commercial anti-cyclic citrullinated peptide 2 (anti-CCP-2) assays, which use mixed cyclic citrullinated peptides as an artificial mimic of the true autoantigens. Although the anti-CCP-2 assay is of high diagnostic importance and is highly specific for RA (14), it does not provide information about which citrullinated proteins are recognized by the anti-CCP-2 antibodies. Moreover, these artificial citrullinated peptides are probably not expressed in the synovial joints. Therefore, the anti-CCP-2 assay may not detect all antibody reactivity with citrullinated antigens known to exist in the joints of patients with RA. Circumstantial evidence from previous studies has suggested that ACPAs with different fine specificities are triggered in different genetic and environmental contexts (15), and that ACPAs with different fine specificities may have different effector functions (16,17). It is therefore of interest to understand the determinants that trigger ACPAs, to elucidate the relationship between patterns of ACPAs and clinical symptoms, and to analyze the patterns of ACPAs in different genetic and environmental contexts.

The importance of determining the fine specificity of ACPAs in RA is supported by a number of studies in which different citrullinated antigens in different organs, as well as different effects of antibodies against these citrullinated antigens, have been noted. Thus, for example, it was recently shown that certain citrullinated proteins (particularly the citrullinated vimentin peptides

CitVim60–75 and CitVim435–455) are present both in the lungs and in the joints of patients with RA (18). Moreover, antibodies with changed glycosylation patterns, such as RFs and ACPAs, differentiate osteoclasts and promote bone loss (19). Further citrullination of the C1 epitope on type II collagen (CitCII) has been identified in RA joints, and murine antibodies to this epitope have been found to induce arthritis in mice (20). Antibodies to several citrullinated peptides are commonly found in the serum of patients with RA, including, but not limited to, peptides from filaggrin (Fil) (21), vimentin (Vim) (22), α -enolase (Eno) (23), α - and β -fibrin (Fib) (24), and CII (25). Interestingly, the frequency of reactivity with certain citrullinated peptides was also found to be significantly increased in RA patients carrying the HLA-DRB1 shared epitope (SE) alleles (26).

Most studies of ACPA fine specificities and their relationship to genetic and environmental determinants in RA have so far been performed in Caucasian populations. In order to investigate the effects of different genetic and environmental determinants on ACPA fine specificity in different RA populations, we utilized a recently developed multiplex analytic microarray system to examine in parallel the antibody reactivity against different peptides derived from citrullinated proteins in RA patients investigated in a case-control study from Malaysia (the Malaysian Epidemiological Investigation of Rheumatoid Arthritis [MyEIRA] study) in comparison with RA patients in a case-control study from Sweden (the Swedish EIRA study), and assessed the relationship between the occurrence of different ACPA patterns and the different genetic backgrounds of the patients in the Malaysian and Swedish populations.

PATIENTS AND METHODS

Study populations. The MyEIRA multicenter case-control study is a sister study to the Swedish EIRA study. The study subjects were recruited between August 2005 and December 2009 throughout peninsular Malaysia (7,27,28). For the present study, we analyzed a total of 1,231 RA cases (Malay, $n = 514$ [41.8%]; Chinese, $n = 255$ [20.7%]; Indian, $n = 404$ [32.8%]; mixed ethnicities, $n = 58$ [4.7%]) and 1,625 healthy controls (Malay, $n = 1,024$ [63.0%]; Chinese, $n = 208$ [12.8%]; Indian, $n = 298$ [18.4%]; mixed ethnicities, $n = 95$ [5.8%]).

The Swedish EIRA study is a large population-based case-control study on early RA in which concomitant information on both environmental/lifestyle factors and genetics was obtained (6,29). For the serologic analyses in the present study, we analyzed data generated between May 1996 and May 2006 from a total of 2,858 RA cases and 578 healthy controls in the Swedish EIRA population. The vast majority of RA patients from the EIRA study were European Caucasians, while fewer than 2% of the study subjects reported having non-Caucasian ancestry.

The design and selection criteria utilized in the MyEIRA and EIRA studies were similar. All patients were individuals who were newly diagnosed as having RA by a rheumatologist, based on the American College of Rheumatology 1987 classification criteria for RA (30). In the MyEIRA study, ~90% of the patients had a diagnosis of early RA (within 3 years of disease onset, $n = 1,090$), while 10% of the patients had longstanding RA (>3 years from disease onset, $n = 141$). The mean \pm SD disease duration in the MyEIRA study was 1.0 ± 2.0 years, 1.2 ± 1.9 years, and 1.1 ± 1.5 years in the Malay, Chinese, and Indian RA patients, respectively. All Swedish RA patients in the EIRA study were enrolled within 12 months after onset of their first symptoms.

All subjects were ages 18–70 years at the time of recruitment. Healthy controls were randomly selected from the respective national populations, with matching for sex, age, and residential area for both studies. Additional information about the MyEIRA and EIRA studies is available in the literature (6,7,27–29,31–35). The characteristics of the study populations are summarized in Table 1.

All participants were informed about the research and gave their written informed consent. Sera for serologic analysis and cells for DNA preparation were collected from all participants at the time of diagnosis in the MyEIRA and EIRA studies. Samples were stored at -80°C until used.

Ethics approval. Both studies were approved by the local ethics committees at each institution (the Medical Research and Ethics Committee of the Ministry of Health Malaysia, and the Stockholm Regional Ethics Committee, Sweden).

Anti-CCP-2 assay. In both the MyEIRA and EIRA studies, anti-CCP-2 levels in the patients' serum were measured using a standard anti-CCP-2 antibody enzyme-linked immunosorbent assay (ELISA) (Immunoscan-RA Mark 2 ELISA test; EuroDiagnostics). Samples with a value of >25 units/ml were considered anti-CCP-2 positive, in accordance with the manufacturer's instructions.

ACPA multiplex assay. Serum samples were analyzed for the presence of IgG-specific ACPAs, using a custom-made microarray based on the ImmunoCAP ISAC system (Phadia AB), which contains 10 different citrullinated peptides and their native arginine-containing counterparts. These citrullinated peptides include Fil307–324 (filaggrin), Vim60–75 (vimentin), Vim2–17 (vimentin), Fib β 36–52 (fibrinogen) (36), Fib α 563–583 (fibrinogen) (36), Fib α 580–600 (fibrinogen) (36), Fib β 62–81a (fibrinogen) (36), Fib β 62–81b (fibrinogen) (36), Eno5–21 (α -enolase), and the triple-helical C1 epitope on CII with citrulline at positions 355 and 378 (GPO)5GLOGA[Cit]OGDAGPQGVGPS (GPO)2 (CitCII355–378) (details on the investigated peptides are available from the corresponding author upon request). A full description of this technology, with extensive validation of the chip-based technique in comparison with ELISA-based technology, and a review of the diagnostic performance of this microarray system was recently published (37). Overall, microarray results were available from a total of 1,231 RA patients in the Malaysian study and 2,858 RA patients in the Swedish study.

In addition, a total of 249 Malaysian healthy control subjects (143 Malays, 41 Chinese, 49 Indian, and 16 of mixed ethnicity) and 578 Swedish healthy control subjects were randomly selected, and their serum samples were tested with the multiplex assay to establish a cutoff value for diagnostic specificity at 98% for each of the 10 citrullinated peptides. Cutoff

values were determined separately for the Malaysian and Swedish cohorts, because we have previously found that there are differences between Malaysian and Swedish subjects with regard to the expression patterns of other antibodies, such as RF isotypes (34) and antibodies against the full CII molecule (Too CL and Manivel VA: unpublished observations), and also because the EIRA and MyEIRA subjects were investigated at different time points.

HLA-DRB1 genotyping. HLA-DRB1 genotyping of DNA from the MyEIRA study subjects was performed as previously described (7). Briefly, high-resolution genotyping of HLA-DRB1 alleles was performed using polymerase chain reaction (PCR) and the sequence-specific oligonucleotide probe hybridization method (LABType HD Class II DRB1*; One Lambda Inc.) with a Luminex Multi-Analyte Profiling System (xMAP), in accordance with the manufacturer's instructions. In the EIRA study subjects, HLA typing was performed using sequence-specific priming-PCR (Olerup SSP) as described elsewhere (38). The HLA-DRB1*01, *04:01, *04:04, *04:05, *04:08, *04:10, and *10 alleles were classified as the SE. HLA-DRB1 information was available for all subjects from the MyEIRA study and for 2,831 RA patients and 566 controls from the EIRA study.

Statistical analysis. The net ACPA results were calculated by subtracting the fluorescence intensity of the arginine-containing control peptide from that of each citrullinated peptide in the serum of all RA patients and controls. These net values were then used to construct receiver operating characteristic (ROC) curves and to compare their diagnostic performance (details available from the corresponding author upon request) (37). This calculation of net ACPA reactivity was performed for all peptides.

To allow stringent comparison of the diagnostic performances of each of the different citrullinated peptides, the cutoff point for each investigation was defined as the fluorescence intensity corresponding to a diagnostic specificity of 98%, calculated from the fluorescence intensity values observed in the 249 randomly selected control subjects in the MyEIRA study and 578 randomly selected control subjects in the EIRA study.

Data evaluation and statistical analysis were performed using SPSS software, version 20.0. Categorical data were analyzed by chi-square test. The 2×2 association test with Yates' correction was used to analyze potential associations between the different autoantibodies and the presence of SE alleles and/or the HLA-DRB1*09:01 allele. Results were expressed as the odds ratio (OR) with 95% confidence interval (95% CI). *P* values less than 0.05 were considered significant. Spearman's rank correlation analyses and Fisher's *r*-to-*z* transformation for the comparison of rho values between Malaysian and Swedish patients were performed using the statistical software package MedCalc (version 15.2.2; MedCalc Software). For comparison between the MyEIRA and EIRA study cohorts, the corrected *P* values (P_{corr}) were obtained by multiplying the observed *P* values by the number of investigated peptides (total of 11 peptides). ROC curves were constructed using SPSS software, version 20.0.

RESULTS

Basic characteristics of the study populations. The characteristics of the RA patients and healthy controls in

Table 1. Characteristics of the RA patients and healthy controls in the MyEIRA and EIRA study populations*

	RA patients	Healthy controls
Total study sample		
Malaysian (MyEIRA)		
All	1,231	1,625
Ethnic group†		
Malay	514	1,029
Chinese	255	208
Indian	404	293
Swedish (EIRA), all SE positive	2,858	578
Malaysian (MyEIRA)		
All	491 (40.0)	263 (16.2)
Ethnic group		
Malay	182 (35.4)	133 (13.0)
Chinese	93 (36.5)	26 (12.5)
Indian	194 (48.0)	88 (30.0)
Swedish (EIRA), all‡	2,084 (73.6)	296 (52.3)
Anti-CCP-2 positive		
Malaysian (MyEIRA)		
All	792 (64.3)	39 (2.4)
Ethnic group		
Malay	311 (60.5)	25 (2.4)
Chinese	169 (66.3)	7 (3.4)
Indian	270 (66.8)	6 (2.0)
Swedish (EIRA), all	1,841 (64.4)	9 (1.6)
ACPA positive (with anti-CCP-2 positive)§		
Malaysian (MyEIRA)		
All	901 (73.2)	42 (16.9)
Ethnic group		
Malay	353 (68.7)	17 (11.9)
Chinese	185 (72.5)	9 (22.0)
Indian	321 (79.5)	12 (24.0)
Swedish (EIRA), all	2,112 (73.9)	87 (15.1)
ACPA positive (without anti-CCP-2 positive)¶		
Malaysian (MyEIRA)		
All	844 (68.6)	40 (16.1)
Ethnic group		
Malay	332 (64.6)	17 (11.9)
Chinese	169 (66.3)	8 (19.5)
Indian	304 (75.2)	11 (22.0)
Swedish (EIRA), all	2,041 (71.4)	82 (14.2)
Anti-CCP-2 positive (without SE alleles)		
Malaysian (MyEIRA)		
All	399 (50.4)	31 (2.3)
Ethnic group		
Malay	170 (51.4)	22 (2.5)
Chinese	91 (56.2)	6 (3.3)
Indian	118 (56.2)	2 (1.0)
Swedish (EIRA), all	274 (36.7)	7 (2.6)

* Values are the number (%) of subjects. SE = HLA-DRB1 shared epitope.

† The total sample size for the Malaysian Epidemiological Investigation of Rheumatoid Arthritis (MyEIRA) study sample included a fourth ethnic group, subjects of mixed ethnicity (n = 58 patients with rheumatoid arthritis [RA] and n = 95 healthy controls) (data not shown).

‡ In the Swedish Epidemiological Investigation of Rheumatoid Arthritis (EIRA) study, serum samples for the HLA-DRB1 genotyping analyses were available from 2,831 RA patients and 566 healthy controls.

§ Anti-citrullinated protein antibody (ACPA) positive (with anti-cyclic citrullinated peptide 2 [anti-CCP-2] positive) refers to the frequency of subjects with antibody reactivity against any citrullinated peptide and/or antibody reactivity against CCP-2 (Malaysian, n = 1,231 RA patients and n = 249 healthy controls; Swedish, n = 2,858 RA patients and n = 578 healthy controls).

¶ ACPA positive (without anti-CCP-2 positive) refers to the frequency of subjects with antibody reactivity against any of the investigated citrullinated peptides but lacking antibody reactivity against CCP-2 (Malaysian, n = 1,231 RA patients and n = 249 healthy controls; Swedish, n = 2,858 RA patients and n = 578 healthy controls).

the MyEIRA and EIRA studies are presented in Table 1. In the MyEIRA study, 86% of RA patients and 88% of controls were female, while in the EIRA study, the proportion of female subjects was 72% in each group. Anti-CCP-2 antibodies were found in the serum of 64% of RA patients in the Malaysian and Swedish populations. Stratification analysis by ethnicity in the MyEIRA study revealed comparable proportions of anti-CCP-2-positive RA patients among those of Malay, Chinese, or Indian ethnicity ($P = 0.095$) (Table 1).

Small, but consistent, differences in the distributions of anti-CCP-2 and ACPA reactivity were observed between the control populations in each study, with higher proportions of healthy controls in the MyEIRA study showing serum positivity for anti-CCP-2 and/or ACPAs. Reactivity with any single ACPA peptide was not uncommon in the control subjects (16.9% of MyEIRA controls and 15.1% of EIRA controls), but in most of these individuals, anti-CCP-2 reactivity did not reach a diagnostically meaningful level, being observed in only 2.4% of anti-CCP-2-positive controls in the MyEIRA study and in 1.6% of anti-CCP-2-positive controls in the Swedish EIRA study (Table 1).

The cumulative frequency of any ACPA, including anti-CCP-2 and/or antibodies to citrullinated peptides from filaggrin, vimentin, fibrinogen, and CII, was recognized to be close to identical between the Malaysian and Swedish RA cases (73.2% versus 73.9%). The mean number of fine specificities of ACPAs in the MyEIRA and EIRA study cohorts was 3.0 and 3.4, respectively. Furthermore, the frequency of at least 1 ACPA fine specificity, without consideration of the results of the anti-CCP-2 test, was comparable between the RA patients in the MyEIRA study and those in the EIRA study (68.6% versus 71.4%) (Table 1). The high level of overlap between anti-CCP-2 reactivity and the sum of reactivity with individual peptides in RA patients confirms the diagnostic performance of the anti-CCP-2 ELISA test as a good proxy for natural citrullinated epitopes. However, we detected ACPAs in 23 Malaysian RA patients and 210 Swedish RA patients whose serum tested negative on the anti-CCP-2 test, which corresponds to a frequency of 2% of the total patients cohort in the MyEIRA study and 7% of the total patients cohort in the EIRA study.

In this study, HLA-DRB1 SE alleles were detected in 40% of the MyEIRA study patients with RA and in 16.2% of the MyEIRA healthy controls, with a preponderance of HLA-DRB1*04:05 and HLA-DRB1*10 (7). In the EIRA study, HLA-DRB1 SE alleles were detected in 73.6% of the RA patients and 52.3% of the control subjects, with a predominance of HLA-DRB1*04:01 and HLA-DRB1*04:04 among the SE alleles (38). Thus, our findings revealed significant differences in the percentage

of individuals carrying HLA-DRB1 SE alleles between the MyEIRA study and the EIRA study, both among RA cases and among healthy controls (each $P < 0.0001$).

It is noteworthy that more than 50% of the Malaysian anti-CCP-2-positive RA patients (regardless of their ethnicity) did not carry any SE alleles (Table 1). Furthermore, a remarkably high frequency of the HLA-DRB1*09:01 allele has been reported in individuals of Asian ancestry (7–15%) (39), but is less common in Europeans. In the current study, 9.9% of the Malaysian RA patients and 8.1% of the Malaysian healthy controls carried the HLA-DRB1*09:01 allele ($P > 0.05$), compared with only 2.9% of the Swedish RA patients and 3.3% of the Swedish healthy controls ($P > 0.05$). However, it is noteworthy that there was no statistically significant difference between the Malaysian population and the Swedish population with regard to the frequency of HLA-DRB1*09:01 in either RA patients or healthy controls.

Features of the ACPA fine specificity profile in the Malaysian and Swedish RA populations. The numbers of RA patients who were positive for autoantibodies against the citrullinated Vim60–75 peptide and CII peptide were significantly increased in the MyEIRA study as compared with the EIRA study ($P_{\text{corr}} = 1.06 \times 10^{-8}$ for Vim60–75; $P_{\text{corr}} = 0.02$ for CitCII355–378) (Figure 1). Conversely, the frequencies of positive reactivity against the citrullinated peptides Vim2–17 ($P_{\text{corr}} = 1.91 \times 10^{-4}$), Fib β 62–81b ($P_{\text{corr}} = 2.47 \times 10^{-22}$), and Eno5–21 ($P_{\text{corr}} = 3.64 \times 10^{-57}$) were significantly decreased in the Malaysian RA population when compared with the Swedish RA population. The percentages of RA patients with antibodies against the other investigated citrullinated peptides, such as Fil307–324, Fib β 36–52, Fib α 563–583, Fib α 580–600, and Fib β 62–81a, were comparable between the MyEIRA and EIRA studies (Figure 1).

With regard to the overall contribution of reactivity with each peptide, the 2 study cohorts had different population profiles for these ACPA peptide reactivities, as illustrated in Figure 1, but there was also a significant overlap. It is notable that with regard to autoantibodies to 2 of the peptides, Fib β 62–81b and Eno5–21, the frequency of antibody-positive individuals in the Swedish RA population was double or more than double that in the Malaysian RA population.

It is noteworthy that when reactivity with the different peptides from the same protein were assessed, we found a higher frequency of autoantibodies to Vim60–75, but a lower frequency of autoantibodies to Vim2–17, in the Malaysian RA population compared with the Swedish RA population. Similarly, heterogeneity in responses to different epitopes was also detected for the fibrinogen peptides. Although the frequency of

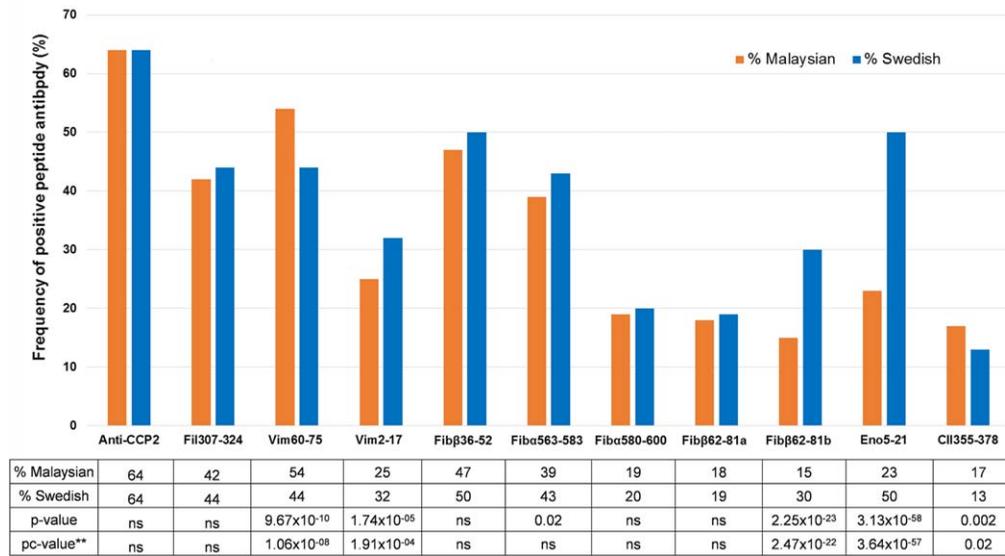


Figure 1. Frequencies of anti-citrullinated protein antibodies (ACPAs) to specific peptides among patients with rheumatoid arthritis in the Malaysian Epidemiological Investigation of Rheumatoid Arthritis study (n = 1,231) and the Swedish Epidemiological Investigation of Rheumatoid Arthritis study (n = 2,858). The corrected P (pc) values were calculated by multiplying the observed P values by the number of investigated peptides (total of 11). Anti-CCP-2=anti-cyclic citrullinated peptide 2; Fil = filaggrin; Vim = vimentin; Fib = fibrinogen; Eno = enolase; CitII = type II collagen; NS = not significant.

autoantibodies to 4 of the 5 investigated fibrinogen peptides was almost equal between the 2 populations, it was significantly different for Fibβ62–81b (Figure 1).

We also investigated the frequencies of ACPA reactivity between the 3 ethnic groups in the MyEIRA study population. Our findings revealed that RA patients of Indian ethnicity, as compared with those of

Malay or Chinese ethnicity, demonstrated increased frequencies of ACPA positivity for 5 of the 10 investigated citrullinated peptide/protein antibodies (details available from the corresponding author upon request).

Comparison of quantitative data on reactivity of each ACPA peptide and anti-CCP-2 positivity. In the quantitative results, the frequencies of all of the

Table 2. Comparison of Spearman’s rank correlations for reactivity with individual ACPA peptide antibodies and positivity for anti-CCP-2 antibodies between the Malaysian and Swedish RA populations*

ACPA peptide antibody	Malaysian RA patients		Swedish RA patients		Comparison of Spearman’s rho values between studies†		
	Spearman’s rho	P	Spearman’s rho	P	z-statistic	P	P _{corr}
Fil307–324	0.733	<0.0001	0.739	<0.0001	–0.3836	0.7012	NS
Vim60–75	0.697‡	<0.0001	0.686	<0.0001	0.6178	0.5367	NS
Vim2–17	0.53‡	<0.0001	0.523	<0.0001	0.2838	0.7766	NS
Fibβ36–52	0.752	<0.0001	0.802	<0.0001	–3.7113	0.0002	0.002
Fibα563–583	0.699	<0.0001	0.742	<0.0001	–2.6248	0.0087	NS
Fibα580–600	0.564	<0.0001	0.602	<0.0001	–1.6878	0.0914	NS
Fibβ62–81a	0.338	<0.0001	0.398	<0.0001	–2.0347	0.0419	NS
Fibβ62–81b	0.469‡	<0.0001	0.556	<0.0001	–3.4647	0.0005	0.005
Eno5–21	0.532‡	<0.0001	0.707	<0.0001	–8.4461	<0.0001	<0.0001
CitII355–378	0.427‡	<0.0001	0.365	<0.0001	2.1561	0.0311	NS

* Reactivity with individual anti-citrullinated protein antibody (ACPA) peptide antibodies was assessed for correlation with positivity for anti-cyclic citrullinated peptide 2 antibodies (anti-CCP-2) in patients with rheumatoid arthritis (RA). These ACPA peptide antibodies included antibodies against filaggrin (Fil), vimentin (Vim), fibrinogen (Fib), enolase (Eno), and citrullinated type II collagen (CitII).

† Comparison of Spearman’s correlation coefficients (rho values) between the 2 studies was done with Fisher’s r-to-z transformation. Corrected P values (P_{corr}) were calculated by multiplying the observed P values by the number of investigated peptides (total of 10 peptides). NS = not significant.

‡ Significant difference versus Swedish patients.

Table 3. Association between HLA-DRB1 SE alleles and/or the HLA-DRB1*09:01 allele and the presence of anti-CCP-2 antibodies in Malaysian and Swedish patients with RA*

	Malaysian study			Swedish study		
	No. of cases/no. of controls	OR	95% CI	No. of cases/no. of controls	OR	95% CI
Anti-CCP-2 positive						
No SE	379/1,283	Referent	–	274/270	Referent	–
Any SE	371/247	5.08	4.17–6.20	1,550/296	5.16	4.19–6.36
SE– and DRB1*09:01–	309/1,176	Referent	–	–	–	–
SE+ and/or DRB1*09:01+	441/354	4.74	3.93–15.72	–	–	–
Anti-CCP-2 negative						
No SE	324/1,283	Referent	–	473/270	Referent	–
Any SE	98/247	1.57	1.21–2.05	534/296	1.03	0.84–1.27
SE– and DRB1*09:01–	295/1,176	Referent	–	–	–	–
SE+ and/or DRB1*09:01+	127/354	1.43	1.13–1.82	–	–	–
Vim60–75 positive						
No SE	276/1,283	Referent	–	131/270	Referent	–
Any SE	353/247	6.64	5.39–8.18	1,103/296	7.68	6.01–9.81
SE– and DRB1*09:01–	228/1,176	Referent	–	–	–	–
SE+ and/or DRB1*09:01+	401/354	5.84	4.78–7.15	–	–	–
Vim60–75 negative						
No SE	427/1,283	Referent	–	616/270	Referent	–
Any SE	116/247	1.41	1.10–1.81	981/296	1.45	1.20–1.76
SE– and DRB1*09:01–	376/1,176	Referent	–	–	–	–
SE+ and/or DRB1*09:01+	167/354	1.48	1.19–1.83	–	–	–
Vim2–17 positive						
No SE	139/1,283	Referent	–	120/270	Referent	–
Any SE	150/247	5.61	4.29–7.33	792/296	6.02	4.67–7.75
SE– and DRB1*09:01–	118/1,176	Referent	–	–	–	–
SE+ and/or DRB1*09:01+	171/354	4.81	3.70–6.26	–	–	–
Vim2–17 negative						
No SE	564/1,283	Referent	–	627/270	Referent	–
Any SE	319/247	2.94	2.42–3.56	1,292/296	1.87	1.55–2.27
SE– and DRB1*09:01–	486/1,176	Referent	–	–	–	–
SE+ and/or DRB1*09:01+	397/354	2.71	2.27–3.24	–	–	–
Fibβ62–81b positive						
No SE	79/1,283	Referent	–	105/270	Referent	–
Any SE	100/247	6.08	4.37–8.39	750/296	6.52	5.01–8.47
SE– and DRB1*09:01–	69/1,176	Referent	–	–	–	–
SE+ and/or DRB1*09:01+	110/354	5.30	3.83–7.32	–	–	–
Fibβ62–81b negative						
No SE	624/1,183	Referent	–	642/270	Referent	–
Any SE	369/247	2.83	2.35–3.42	1,324/296	1.90	1.57–2.29
SE– and DRB1*09:01–	535/1,176	Referent	–	–	–	–
SE+ and/or DRB1*09:01+	458/354	2.84	2.39–3.38	–	–	–
Eno5–21 positive						
No SE	85/1,283	Referent	–	184/270	Referent	–
Any SE	180/247	11.00	8.22–14.73	1,227/296	6.08	4.85–7.63
SE– and DRB1*09:01–	75/1,176	Referent	–	–	–	–
SE+ and/or DRB1*09:01+	190/354	8.42	6.28–11.28	–	–	–
Eno5–21 negative						
No SE	618/1,283	Referent	–	563/270	Referent	–
Any SE	289/247	2.43	2.00–2.95	857/296	1.39	1.14–1.69
SE– and DRB1*09:01–	529/1,176	Referent	–	–	–	–
SE+ and/or DRB1*09:01+	378/354	2.37	1.99–2.84	–	–	–
CitCII355–378 positive						
No SE	90/1,283	Referent	–	65/270	Referent	–
Any SE	103/247	5.94	4.34–8.14	312/296	4.38	3.20–5.99
SE– and DRB1*09:01–	80/1,176	Referent	–	–	–	–
SE+ and/or DRB1*09:01+	113/354	4.69	3.44–6.40	–	–	–
CitCII355–378 negative						
No SE	613/1,283	Referent	–	682/270	Referent	–
Any SE	366/247	3.10	2.57–3.74	1,772/296	2.37	1.97–2.86
SE– and DRB1*09:01–	524/1,176	Referent	–	–	–	–
SE+ and/or DRB1*09:01+	455/354	2.88	2.43–3.43	–	–	–

* SE = shared epitope; OR = odds ratio; 95% CI = 95% confidence interval (see Table 2 for other definitions).

Table 4. Frequency of ACPA peptide antibody positivity in the Malaysian and Swedish populations of RA patients who were positive or negative for the HLA-DRB1 SE*

ACPA peptide antibody	SE-negative RA		SE-positive RA	
	Malaysian (n = 703)	Swedish (n = 747)	Malaysian (n = 469)	Swedish (n = 2,084)
Anti-CCP-2	379 (53.9)	274 (36.7)	371 (79.1)	1,550 (74.4)
Fil307-324	266 (37.8)	208 (27.8)	230 (49.0)	1,028 (49.3)
Vim60-75	274 (39.0)	131 (17.5)	353 (75.3)	1,103 (52.9)
Vim2-17	139 (19.8)	120 (16.1)	150 (32.0)	792 (38.0)
Fibβ36-52	274 (39.0)	224 (30.0)	267 (56.9)	1,191 (57.1)
Fibα563-583	220 (31.3)	166 (22.2)	231 (49.3)	1,045 (50.1)
Fibα580-600	96 (13.7)	75 (10.0)	124 (26.4)	499 (23.9)
Fibβ62-81a	113 (16.1)	108 (14.5)	98 (20.9)	428 (20.5)
Fibβ62-81b	79 (11.2)	105 (14.1)	100 (21.3)	750 (36.0)
Eno5-21	85 (12.1)	184 (24.6)	180 (38.4)	1,227 (58.9)
CitCII355-378	90 (12.8)	65 (8.7)	103 (22.0)	312 (15.0)

* Values are the number (%) of RA patients who were negative or positive for the HLA-DRB1 shared epitope (SE) and whose serum showed reactivity to the listed ACPA peptide antibodies. See Table 2 for other definitions.

ACPA peptide antibodies investigated were significantly correlated with the frequency of anti-CCP-2 antibody positivity ($P < 0.0001$) in all Malaysian and Swedish RA patients; the Spearman's correlation coefficients (rho values) ranged from 0.338 to 0.752 in the MyEIRA study and 0.365 to 0.802 in the EIRA study (Table 2). Interestingly, the best correlation with anti-CCP-2 antibody positivity was observed for Fibβ36-52, both in the Malaysian RA population and in the Swedish RA population. Comparison of the Spearman's rank correlation coefficients between the MyEIRA and EIRA studies revealed significant population differences in correlations for 3 of the 10 ACPA peptide antibodies (Fibβ36-52, Fibβ62-81b, and Eno5-21) (Table 2). These findings partly overlap with the findings of significantly different sensitivities of each peptide between Malaysian and Swedish RA patients (Figure 1).

Association between the fine specificity of the ACPA response and HLA-DRB1 SE/HLA-DRB1*09:01 alleles in the Malaysian RA patients and HLA-DRB1 SE alleles in the Swedish RA patients. When we used the anti-CCP-2 assay as a reference for ACPA positivity, we observed a consistent pattern of association between HLA-DRB1 SE alleles and ACPA reactivity. The OR for an association between the presence of HLA-DRB1 SE alleles and all ACPAs was increased in the anti-CCP-2-positive subsets of patients with RA, whereas a modest-to-weak association was noted in the anti-CCP-2-negative RA subsets (Table 3 and details available from the corresponding author upon request). It is also noteworthy that the OR for the association of HLA-DRB1 SE alleles with anti-Eno5-21 reactivity in

Malaysian RA patients (OR 11.00, 95% CI 8.22-14.73) did not overlap with the corresponding values in the Swedish RA population (OR 6.08, 95% CI 4.85-7.63) (Table 3).

Since the HLA-DRB1*09:01 allele association with RA susceptibility is known to be evident in Asian populations, we performed additional association analyses in which we assessed associations with the combined presence of HLA-DRB1 SE alleles and/or the HLA-DRB1*09:01 allele in the MyEIRA study. Overall, in the Malaysian population, irrespective of ethnicity, the combined genetic effect size of HLA-DRB1 SE alleles and/or HLA-DRB1*09:01 was comparable to that of the presence of any HLA-DRB1 SE allele alone with regard to the risk of developing ACPA-positive RA (Table 3 and details available from the corresponding author upon request).

Furthermore, we also assessed the reactivity against CCP-2 and reactivity against each individual citrullinated peptide in analyses stratified according to HLA-DRB1 SE status in both the MyEIRA study and the Swedish EIRA study. Antibodies against the Vim60-75 peptide (frequency of 75.3% among RA patients in the MyEIRA study) seemed to account for most of the anti-CCP-2 reactivity in the Malaysian RA patients who were positive for the HLA-DRB1 SE, regardless of ethnicity (Table 4), whereas reactivities to other peptides showed a weaker relationship to the presence of the HLA-DRB1 SE alleles. In contrast, in the Swedish EIRA study, the presence of the majority of autoantibodies to the investigated citrullinated peptides was correlated with the presence of the HLA-DRB1 SE alleles in RA patients.

Levels of peptide reactivity in anti-CCP-2-positive versus anti-CCP-2-negative RA patients. The recognition of multiple ACPA specificities was found mainly among anti-CCP-2-positive RA patients (details available from the corresponding author upon request). Anti-CCP-2-positive RA patients were grouped according to the levels of peptide reactivity, as follows: 1) low-to-moderate levels (<1,600 units/ml), comprising 644 patients (81.3%) in the MyEIRA study and 1,469 patients (79.8%) in the EIRA study, and 2) high levels ($\geq 1,600$ units/ml), comprising 148 patients (18.7%) in the MyEIRA study and 372 patients (20.2%) in the EIRA study. Based on these groups, the fractions of RA patients with high levels of anti-CCP-2 reactivity did not differ between the MyEIRA study and the EIRA study ($P = 0.4$).

In addition, when the Spearman's rank correlation test was performed to assess correlations between the number of detected ACPA specificities and the net

Table 5. Frequency of ACPA specificities among RA patients in the Malaysian study who were ever smokers compared with nonsmokers*

ACPA peptide antibody	Ever smoker (n = 90)		Nonsmoker (n = 1,017)		P
	Peptide positive	Peptide negative	Peptide positive	Peptide negative	
Anti-CCP-2	72 (80)	18 (20)	629 (61.8)	388 (38.2)	0.0009
Fil307-324	45 (50)	45 (50)	408 (40.1)	609 (59.9)	NS
Vim60-75	26 (28.9)	64 (71.1)	249 (24.5)	768 (75.5)	NS
Vim2-17	61 (67.8)	29 (32.2)	526 (51.7)	491 (48.3)	0.004
Fib β 36-52	50 (55.6)	40 (44.4)	455 (44.7)	562 (55.3)	NS
Fib α 563-583	50 (55.6)	40 (44.4)	360 (35.4)	657 (64.6)	0.0002
Fib α 580-600	26 (28.9)	64 (71.1)	184 (18.1)	833 (81.9)	0.01
Fib β 62-81a	25 (27.8)	65 (72.2)	175 (17.2)	842 (82.8)	0.01
Fib β 62-81b	16 (17.8)	74 (82.2)	148 (14.6)	869 (85.4)	NS
Eno5-21	36 (40)	54 (60)	207 (20.4)	810 (79.6)	<0.0001
CitIII355-378	17 (18.9)	73 (81.1)	171 (16.8)	846 (83.2)	NS

* Information on smoking was missing for 124 RA patients. Values are the number (%). See Table 2 for definitions.

reactivity value of anti-CCP-2, we found similar correlations in each study: $r^2 = 0.657$ ($P < 0.0001$) in the MyEIRA study, and $r^2 = 0.691$ ($P < 0.0001$) in the EIRA study (data not shown). These findings indicate that in the EIRA study, enrichment in the level of reactivity could not be attributed to higher absolute numbers of individual autoantibodies.

Frequency of ACPA specificities in ever smokers versus nonsmokers in the Malaysian RA population.

In the MyEIRA study, only 90 (8.1%) of the 1,107 Malaysian RA patients reported a history of ever smoking. The percentage of patients in the MyEIRA study who were ever smokers compared with nonsmokers among those who were positive or negative for each peptide antibody are presented in Table 5. The results demonstrated significant differences in the numbers of patients with specific ACPAs between ever smokers and nonsmokers for 6 of the 11 studied ACPA specificities, including anti-CCP-2, Vim2-17, Fib α 563-583, Fib α 580-600, Fib β 62-81a, and Eno5-21 (Table 5).

DISCUSSION

In the present study, we observed profound differences in aspects of ACPA fine specificity between 2 cohorts of RA patients from Malaysia and Sweden, in which the same classification criteria had been used for patient inclusion and in which identical criteria (98% specificity for each peptide, use of matched population controls) were used to establish cutoff values. Thus, despite the fact that the general ACPA multiplex assay showed results identical to those of the anti-CCP-2 test in terms of the proportions of patients positive for ACPAs, we found different frequencies of autoantibodies for 5 of the 10 investigated peptides.

We also confirmed that the occurrence of ACPAs in general was dependent on the presence of SE alleles, but also noted intriguing ethnicity-specific differences, since we found that the relationship between the presence of HLA-DRB1 SE alleles and specific ACPAs differed between Malaysian and Swedish RA patients.

Publically available information regarding the detection of specific ACPAs in different ethnic groups is very limited, and the present study is, to our knowledge, the first to systematically compare the occurrence of ACPA fine specificities in 2 populations in which different genetic and environmental factors may influence the occurrence of ACPAs, as well as the occurrence of RA. In this context, it is interesting that these RA populations in Malaysia and Sweden, both of which were composed of patients with disease of recent onset, show almost identical frequencies of anti-CCP-2-positive individuals.

What is the reason for differing autoantibody production in different populations? Most probably it is a consequence of both genetic and environmental factors, which most likely act in synergy (6). There are vast differences between the European and Asian lifestyles and environmental exposures. However, the outcomes of RA, expressed as the clinical manifestations of RA, the course of the disease, and the overall involvement of autoimmunity in RA, are very similar in the 2 populations, and both patient cohorts were classified according to the same disease classification criteria. The estimated prevalence of RA in different countries is ~0.5-1%, and it is very similar in Sweden and Malaysia (40-42).

Notably, the most well-described environmental risk factor for RA—smoking—is a risk factor for ACPA-positive RA in both the Malaysian and Swedish populations (27,43). Whereas a status of current or, in particular,

previous smoking is common in the Swedish RA population (6), only a minor fraction of Malaysian RA patients (mostly men) are smokers (27). The smoking prevalence among women in Malaysia is very low, being 1% among female patients with RA and lower than 0.4% among female control subjects, although >80% of the RA patients in the MyEIRA study are women. Therefore, we believe that smoking does not contribute to the high prevalence of ACPAs among female Malaysian RA patients.

In this study, HLA-DRB1*04:05 and HLA-DRB1*10 were found to be the most common alleles in the Malaysian RA patients, while HLA-DRB1*04:01 and HLA-DRB1*04:04 were prominent in the Swedish RA patients. Since the distribution of specific HLA-DRB1 SE alleles varied between the Malaysian and Swedish populations, we hypothesized that the associations between specific HLA-DRB1 SE alleles and ACPA specificity might also differ between populations because their allele frequencies are different. This could be one of the possible explanations for the differences in autoantibody frequencies observed in the present study. It is also noteworthy that the influence of genetic factors differed between the 2 populations, as indicated by the high occurrence of ACPAs among Malaysian RA patients who were negative for HLA-DRB1 SE alleles (Malays 51.4%, Chinese 56.2%, and Indian 56.2%). Therefore, the most interesting results of the present study are that the total frequencies of ACPAs, both in the RA populations and in the similarly matched healthy control populations, in Sweden and Malaysia are so similar, despite the fact that the respective pools of ACPAs consisted of antibodies with different fine specificities that were generated in different genetic and environmental contexts.

Our study also indicates that a significant number of anti-CCP-2-negative RA patients were found to have ACPAs when they were detected as reactivity with individual natural peptides (2% of the MyEIRA study patients and 7% of the EIRA study patients). This observation points toward a possible source of misclassification of autoantibody-positive and antibody-negative RA patients, and suggests that the anti-CCP assay could be further improved. Importantly, this may influence the diagnosis of early RA.

Our data support previous findings that have suggested that different citrullinated proteins may be involved in autoimmune reactions in RA. Moreover, the contribution of these proteins, including different epitopes, does not follow a single pattern and is so complex that it may suggest randomness in this process, with more abundant proteins being more likely involved in the development of antibodies against citrullinated epitopes in RA. It is evident from the analyses of fibrinogen and vimentin peptides

in our study that different human RA populations differ with regard to their dominant ACPA epitopes. Although our findings were based on the immune response to autoantigens, similar mechanisms could also be at work in the response to infectious agents and vaccines.

This hypothesis obviously needs much more empirical support. Nevertheless, we believe that the multiethnic and multicultural approach to the etiology and molecular pathogenesis of RA applied in the present study may be a useful complement to other, more mechanistic studies focused on the patterns of antibodies with fine specificities similar to those seen in both Malaysia and Sweden.

In conclusion, using a custom-made peptide-based microarray, we have demonstrated significant differences in the occurrence of specific ACPAs between 2 distinct RA populations from Malaysia and Sweden. We hypothesize that the ACPA epitope differences observed could be attributed to genetic and environmental differences between the 2 populations. Such geographically determined epitope differences may help in elucidating the putative causative disease pathways of RA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Too had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Too, Murad, Hansson, Alm, Dhaliwal, Holmdahl, Jakobsson, Alfredsson, Klareskog, Rönnelid, Padyukov.

Acquisition of data. Too, Rönnelid, Padyukov.

Analysis and interpretation of data. Too, Murad, Hansson, Alm, Dhaliwal, Holmdahl, Jakobsson, Alfredsson, Klareskog, Rönnelid, Padyukov.

ADDITIONAL DISCLOSURE

Dr. Alm is an employee of Thermo Fisher Scientific, Uppsala, Sweden.

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