

EXTENDED REPORT

Functional haplotypes of PADI4: relevance for rheumatoid arthritis specific synovial intracellular citrullinated proteins and anticitrullinated protein antibodies

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Ann Rheum Dis 2005;64:1316–1320. doi: 10.1136/ard.2004.033548

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Accepted 7 February 2005
Published Online First
10 March

Background: Haplotypes of PADI4, encoding for a citrullinating enzyme, were associated with rheumatoid arthritis in a Japanese population. It was suggested they were related to the presence of anticitrullinated protein antibodies (ACPA).

Objective: To explore the relation between PADI4 haplotypes, the presence of rheumatoid arthritis specific intracellular citrullinated proteins in synovial membrane, and serum ACPA titres.

Methods: Synovial biopsies and peripheral blood samples were obtained in 59 patients with rheumatoid arthritis. Synovial intracellular citrullinated proteins were detected by immunohistochemistry. Serum ACPA titres were measured by anti-CCP2 ELISA. PADI4 haplotypes were determined by direct sequencing of the four exonic PADI4 single nucleotide polymorphisms.

Results: PADI4 haplotype frequencies and the presence of synovial intracellular citrullinated proteins and ACPA were comparable with previous studies. There was no significant association between PADI4 haplotype 1 or 2 and the presence of synovial intracellular citrullinated proteins, although these proteins were associated with higher serum ACPA. There was no correlation between PADI4 haplotypes and serum ACPA, either by continuous analysis using the titres or by dichotomous analysis using the diagnostic cut off. Further analyses in homozygotes for haplotype 1 or 2 or in heterozygotes (1/2) also failed to show an association between PADI4 polymorphisms and ACPA. This contrasted with the clear association between ACPA levels and HLA-DR shared epitope.

Conclusions: The link between synovial intracellular citrullinated proteins and ACPA emphasises the role of deimination of synovial proteins in rheumatoid arthritis, but the biological relevance of the PADI4 haplotypes for this autoimmune process is questionable, at least in a European population.

Anticitrullinated protein antibodies (ACPA) have been documented extensively over recent years as highly specific serological markers for rheumatoid arthritis, with important clinical implication for diagnosis and prognosis.¹ Although the pathophysiology of ACPA induction and the role of these antibodies in the pathogenesis of rheumatoid arthritis remains to be elucidated, it has been shown that post-translational modification of arginine containing epitopes by deimination (citrullination) is a crucial step in the generation of antigenic targets for ACPA.^{2,3} The citrullination process is mediated by the peptidylarginine deiminase (PAD) enzymes, of which subtypes 2 and 4 are found in human synovial tissue, the primary disease target of rheumatoid arthritis.⁴ In this context, the recent description of an association between rheumatoid arthritis and a functional haplotype of PADI4, the gene encoding for PAD type 4, is of major interest.⁵ Indeed, Suzuki *et al*⁵ identified 17 single nucleotide polymorphisms (SNPs) in the PADI4 gene, of which eight were associated with rheumatoid arthritis in a case-control study. Two haplotypes defined by these SNPs comprised more than 85% of the total number of haplotypes and could be segregated from each other and from the vast majority of other less frequent haplotypes by four exonic SNPs. One of these haplotypes (haplotype 2) was observed more frequently in the rheumatoid group, whereas the other (haplotype 1) was overrepresented in the control group. They indicated that rheumatoid patients homozygous for haplotype 2 were more often positive for ACPA than the two other genotypes (homozygous for haplotype 1 and heterozygous)

and suggested that this might be related to haplotype dependent degree of citrullination of proteins in the synovial membrane.⁵ This hypothesis would fit with our previous demonstration of the rheumatoid arthritis specific presence of intracellular citrullinated proteins in synovium and the co-localisation of these proteins with ACPA reactivity, suggesting a role for the proteins in the humoral autoimmune process.⁶ In order to assess the biological relevance of PADI4 polymorphisms in this process, we investigated directly the link between the described PADI4 haplotypes, rheumatoid arthritis specific synovial intracellular citrullinated proteins, and ACPA.

METHODS

Patients

The study involved 59 patients with rheumatoid arthritis fulfilling the ACR criteria⁷; 59% were female, and their mean (SD) age was 55.5 (15.4) years. All patients had active disease, with a mean serum C reactive protein concentration of 5.2 (5.4) mg/l, mean erythrocyte sedimentation rate (ESR) of 42.6 (24.9) mm/h, and mean swollen joint count of 7.7 (6.0). Rheumatoid factor (assessed by the Waaler-Rose test, cut off level at 1/160) was positive in 61.4% and the HLA-DR shared epitope in 83.1% of the patients. Current treatment included disease modifying antirheumatic drugs in 56.9%.

Abbreviations: ACPA, anticitrullinated protein antibodies; ACR, American College of Rheumatology; PAD, peptidylarginine deiminase; SE, shared epitope; SNP, single nucleotide polymorphism

corticosteroids in 43.5%, and non-steroidal anti-inflammatory drugs in 86%. All patients gave written informed consent for their participation in the study, which was approved by the ethics committee of Ghent University Hospital.

Synovial tissue analysis

All patients had active synovitis of at least one knee joint, which was biopsied by needle arthroscopy. In each patient, eight biopsies were obtained throughout the joint and frozen en block to obtain a representative picture of the synovial membrane. Frozen sections of these synovial biopsies (four sections for each sample) were stained by immunohistochemistry to detect intracellular citrullinated proteins, using a rabbit anti-L-citrulline polyclonal antibody (Biogenesis, Poole, UK) as described previously.⁵⁻⁶ Stained sections were blinded and scored by two independent observers. In two of the 59 samples, tissue sections were of insufficient quality for correct interpretation.

ACPA measurement

Peripheral blood samples were obtained at the time of synovial biopsy sampling. ACPA serum titres were measured by the anti-CCP2 enzyme linked immunosorbent assay (ELISA) containing synthetic citrullinated peptides as substrate (Immunoscan RA, mark 2, Eurodiagnostica, Arnhem, Netherlands). The test was performed according to the manufacturer's instructions. For dichotomous analysis, we used a diagnostic cut off of 42 U/ml which was previously shown to correspond with a 98.6% specificity level.⁸

PADI4 genotyping

DNA extraction was carried out using the genomic DNA purification kit (Puregene, Gentra, Minneapolis, Minnesota, USA). Four exonic PADI4 SNPs (padi4_89*G/A, padi4_90*T/C, padi4_92*G/C, and padi4_104*T/C) were genotyped by direct sequencing. These SNPs were selected not only because they are exonic but also because they segregate the susceptible and non-susceptible functional haplotypes of PADI4 as defined by Suzuki *et al.*⁵ They correspond also to those used by Barton *et al* to define haplotype 1 and 2 in a white UK population.⁹ Briefly, polymerase chain reaction was undertaken using the following primer sets (Invitrogen, San Diego, California, USA): 5'-TTGTCCACAGCTGTGCC-3' and 5'-ACACTGCCACCCACAG-3' for padi4_89 and padi4_90; 5'-GTTTCAGATTTCATACTACGGACC-3' and 5'-GGGATGAGACGGCACTC-3' for padi4_92; and 5'-GACCTGCCATTTCAGGCCAG-3' and 5'-GAATACGTGGGACAGGCCAGC-3' for padi4_104. Sequencing reactions were carried out using the Bigdye Terminator v3.1 sequencing reagents and the ABI PRISM 3100 genetic analyser (Applied Biosystems, Foster City, California, USA).

HLA-DR shared epitope genotyping

HLA-DR shared epitope (SE) was determined using the INNO-LiPA HLA-DRB1 or -DRB decoder amplification kits (Innogenetics, Gent, Belgium) according to the manufacturer's instructions. The following HLA-DR alleles were considered to have the SE: HLA-DRB1*0101, 0401, 0404, and 1001. HLA-DRB1*0405, 0408, and 1402 were not found in our population.

Statistics

A χ^2 test was used for comparisons between two discrete variables. Comparison of serum ACPA levels (not normally distributed; median values and 25th to 75th centiles are given) was assessed by the Mann-Whitney U test. A probability (p) value of less than 0.05 was considered significant.

RESULTS

PADI4 haplotypes frequencies

We determined the four exonic SNPs (padi4_89, padi4_90, padi4_92, and padi4_104) of the PADI4 gene in order to segregate PADI4 haplotype 1 and PADI4 haplotype 2 from other minor haplotypes of PADI4, as described by Suzuki *et al.*⁵ Two of these exonic SNPs, padi4_92 and padi4_104, were associated with rheumatoid arthritis in the Japanese cohort, and the polymorphisms of padi4_89, padi4_90, and padi4_92 resulted in amino acid substitutions. Of the 59 patients included in the present study, one could not be genotyped. Haplotype 1 occurred in 51.7%, haplotype 2 in 34.5%, haplotype 4 (as defined by Suzuki *et al*) in 9.5%, and other haplotypes in 4.3% (table 1). The haplotype frequencies in our cohort closely resemble those reported by Suzuki *et al* in a Japanese population⁵ and by Barton *et al* in a UK population.⁹ This finding supports the assumption that the haplotypes defined by us and by Barton *et al*⁹ by analysing only the four exonic SNPs correspond to those described by Suzuki in a different population.⁵ However, this cannot be demonstrated formally without full haplotype analysis which allows one to distinguish more precisely haplotype 1 and 2 from other haplotypes occurring at low frequencies. In the remainder of the study we focus on haplotype 1 (n = 60) and haplotype 2 (n = 40), which are defined similarly in the three studies and were identified as the non-susceptible and the susceptible haplotype, respectively, in the Japanese cohort.

PADI4 haplotypes are not associated with synovial intracellular citrullinated proteins

Concordant with previous data,⁶ intracellular citrullinated proteins were detected in 45.6% (26/57) of the rheumatoid arthritis synovial tissue samples. Although it is clear that not all synovial citrullinated proteins are rheumatoid arthritis specific,¹⁰⁻¹¹ we and others indicated previously that the synovial intracellular citrullinated targets recognised by the Biogenesis antibody are rheumatoid arthritis specific.¹⁰⁻¹⁴ Furthermore, the co-localisation with ACPA reactivity but not with iNOS⁶ indicates specific staining of citrullinated proteins rather than non-specific staining or staining of free citrulline. This was further confirmed in the present study by the fact that serum ACPA titres were higher in the synovial intracellular citrullinated protein positive group (median 982 U/ml (25th to 75th centiles: 303 to 1800 U/ml)) than in the negative group (median 343 (4 to 963) U/ml) (p = 0.007), emphasising their relevance as antigenic targets for ACPA. Therefore, we next analysed whether PADI4 haplotypes were associated with the synovial expression of these proteins, as suggested by Suzuki *et al.*⁵ Synovial intracellular citrullinated proteins were present in 47.4% of the samples from the PADI4 haplotype 1 group and in 40.5% of those from the PADI4 haplotype 2 group (fig 1A). Moreover, there were no significant differences between haplotype 1 homozygotes, heterozygotes, and haplotype 2 homozygotes (50.0%, 41.7%, and 25.0%, respectively) (fig 1C). Thus the presence of

Table 1 PADI4 haplotype frequencies in a cohort of 58 patients with rheumatoid arthritis, as determined by the four exonic single nucleotide polymorphism of PADI4

Haplotype	Haplotype frequency	SNP			
		89	90	92	104
Haplotype 1	51.7% (60/116)	A	C	C	C
Haplotype 2	34.5% (40/116)	G	T	G	T
Haplotype 4	9.5% (11/116)	G	T	G	C
Others	4.3% (5/116)	NA	NA	NA	NA

NA, not applicable; SNP, single nucleotide polymorphism.

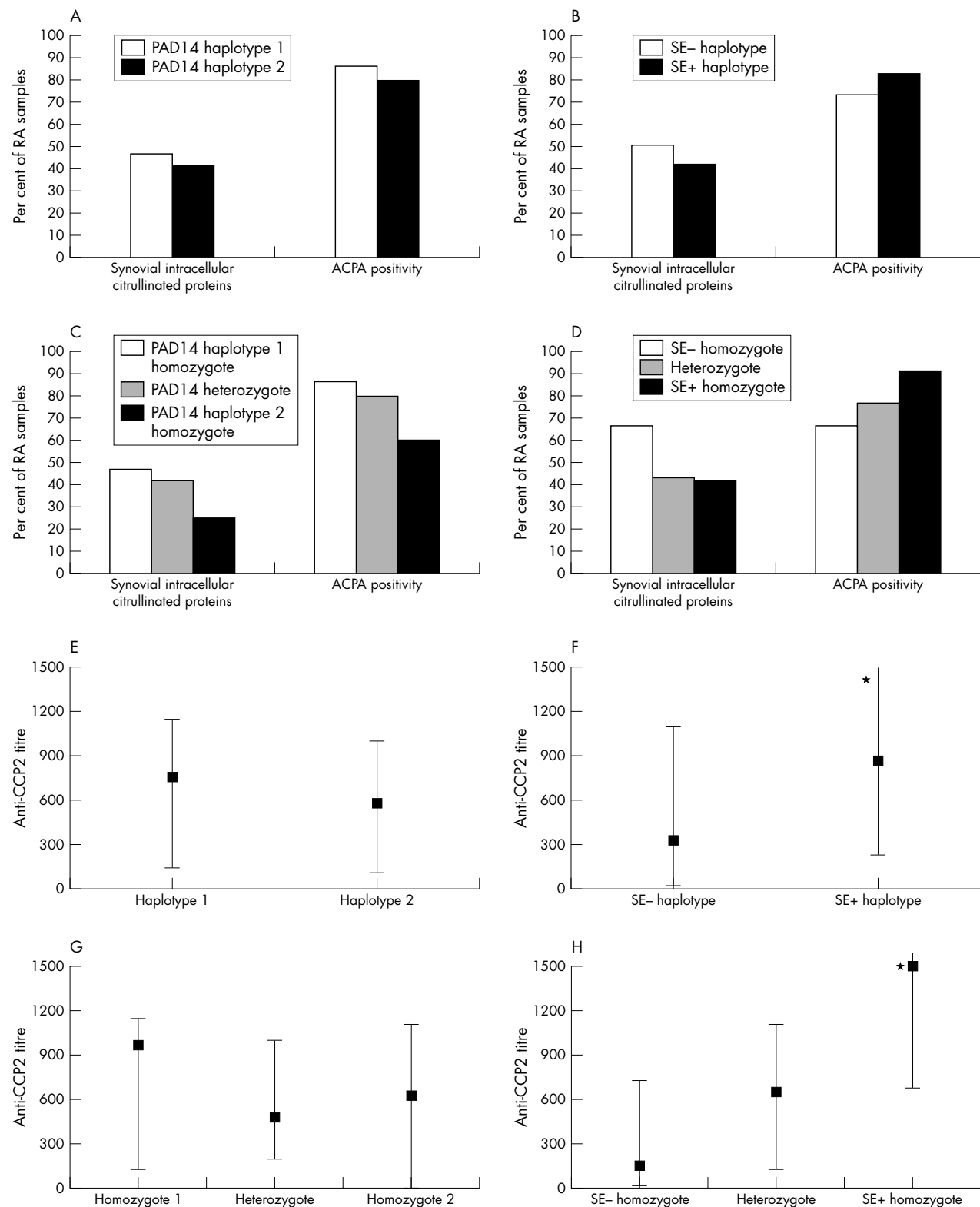


Figure 1 Analysing a cohort of 58 patients with rheumatoid arthritis (RA), we assessed the influence of PADI4 haplotypes on the presence of synovial intracellular citrullinated proteins, positivity for serum anticitrullinated protein antibodies (ACPA), and serum ACPA levels (panels A, C, E, and G). For comparison, a similar analysis was carried out for the HLA-DR shared epitope (SE) (panels B, D, F, and H). Positivity (% of samples) for synovial intracellular citrullinated proteins and ACPA is shown as a function of haplotype (panels A and B) and genotype (panels C and D). Similarly, ACPA levels (units/ml as determined by the anti-CCP2 ELISA; results are represented as median and 25 to 75 centile) are shown as a function of haplotype (panels E and F) and genotype (panels G and H). ELISA, enzyme linked immunosorbent assay. * $p < 0.05$.

rheumatoid arthritis specific intracellular citrullinated proteins in the synovial membrane, which was significantly associated with ACPA titres, was not related to the two main PADI4 haplotypes.

PADI4 haplotypes are not associated with ACPA

As we cannot exclude the possibility that the PADI4 haplotypes are linked with the deimination of other synovial targets of ACPA, such as fibrinogen or vimentin,^{15–16} we next investigated directly the association between PADI4 haplotypes and serum ACPA titres, irrespective of the presence or absence of specific citrullinated antigens in the synovial membrane. There was no difference in ACPA titres between haplotype 1 (median 750 U/ml (25th to 75th centiles: 140 to 1149 U/ml)) and haplotype 2 (629 (102 to 1000) U/ml) ($p = 0.624$) (fig 1E). Similarly, serum ACPA levels were not significantly different between haplotype 1 homozygotes (973 (133 to 1144) U/ml), heterozygotes (487 (207 to 966) U/ml), and haplotype 2 homozygotes (629 (7 to 1018) U/ml) (fig 1G).

Finally, a diagnostic cut off of 42 U/ml, which was previously shown to correspond to a 98.5% specificity level,⁸ was used to define ACPA positive and ACPA negative samples for dichotomous analysis. This also failed to show significant differences between haplotype 1 and 2 (81.7% *v* 77.5% ACPA positive samples) and between haplotype 1 homozygotes (86.7%), haplotype 1 heterozygotes (80%), and haplotype 2 homozygotes (60%) (fig 1, panels A and C). In comparison with the data of Suzuki *et al.*,⁵ we observed more ACPA positive samples in the pooled haplotype 1 homozygotes and heterozygotes (84.6% *v* 50%, $p = 0.05$) and fewer ACPA positive samples in the haplotype 2 homozygotes (60% *v* 87%, $p = 0.09$).

HLA-DR SE haplotypes are associated with ACPA

As the HLA-DR shared epitope may play a role in ACPA induction,¹⁷ we determined for comparison the relation between SE on the one hand and the synovial intracellular citrullinated proteins and ACPA on the other. As for PADI4, there was no significant differences in synovial intracellular citrullinated protein positivity between SE– (50.9%) and SE+ haplotypes (42.4%) nor between SE– homozygotes (66.7%), SE– heterozygotes (42.9%), and SE+ homozygotes (41.7%) (fig 1, panels B and D).

As for ACPA, a dichotomous analysis using the diagnostic cut off showed no difference between SE– haplotypes (73.6%) and SE+ haplotypes (83.1%) but there was a trend towards increased ACPA positivity from SE– homozygotes (66.7%) over SE– heterozygotes (77.1%) to SE+ homozygotes (91.7%) (fig 1, panels B and D). This was confirmed by the continuous analysis of the ACPA levels, which were increased in SE+ haplotypes (865 (252 to 2008) U/ml) *v* SE– haplotypes (332 (29 to 1101) U/ml) ($p = 0.025$) as well as in SE+ homozygotes (1517 (681, 3810) U/ml) compared with heterozygotes (655 (149 to 1107) U/ml) ($p = 0.036$) and SE– homozygotes (152 (18 to 488) U/ml) ($p = 0.019$) (fig 1, panels F and H). As SE is thus significantly associated with ACPA levels and so may bias the analysis of PADI4 haplotypes, we reanalysed the PADI4 data after exclusion of the HLA-DR shared epitope negative patients; again we could not show any difference in serum ACPA titres (continuous analysis) or positivity (dichotomous analysis) in function of PADI4 haplotypes (data not shown).

DISCUSSION

Considering the crucial role of deimination in the generation of antigenic targets for the highly rheumatoid specific ACPA,^{2–3} the association of rheumatoid arthritis with polymorphisms of PADI4 coding for a citrullinating enzyme is

potentially of major interest.⁵ Indeed, the PADI4 haplotype 2—which is the susceptible haplotype in the Japanese study and leads to increased mRNA stability *in vitro*—may be associated with an increased citrullination of synovial proteins, which may in turn induce ACPA. The present study explored this hypothesis, focusing essentially on the previously described rheumatoid specific synovial intracellular citrullinated proteins.⁶ Although the present cohort was representative of previously investigated cohorts in terms of PADI4 haplotype frequencies,^{5–9} presence of synovial intracellular citrullinated proteins,⁶ and ACPA,^{1–8} we were unable to show a link between PADI4 haplotypes on the one hand and rheumatoid specific synovial intracellular citrullinated proteins and ACPA on the other.

With regard to PADI4 and deimination of potential ACPA targets, in the present study we analysed synovial intracellular citrullinated proteins rather than other candidate targets as first, they are highly specific for rheumatoid arthritis^{6–10–14}; second, they co-localise with ACPA reactivity⁶; and third, their presence is associated with significantly higher serum ACPA titres (personal observations and present study). This contrasts with other synovial citrullinated proteins which are not specific for rheumatoid arthritis and which did not show a correlation with ACPA levels.^{10–11} Taken together, these data strongly support a role for the rheumatoid specific synovial intracellular citrullinated proteins detected in this study as antigenic targets for the humoral autoimmune process described. While we could not show a link between synovial intracellular citrullinated proteins and PADI4 haplotypes, it remains possible that this analysis was partially biased by an underestimation of the presence of intracellular citrullinated proteins in the synovial membrane owing to sampling error (either in a single joint or between different joints in a single patient). However, this underestimation should then also apply to the relation between intracellular citrullinated proteins and ACPA titres. Alternatively, it cannot be excluded that other synovial targets of ACPA, such as citrullinated fibrinogen or citrullinated vimentin,^{10–15–16} are associated with PADI4 haplotypes. The fact that neither rheumatoid arthritis specificity nor a direct relation with ACPA titres has yet been demonstrated for these targets does not exclude the possibility that they may play a role in amplifying or perpetuating the synovial citrullinated protein/ACPA conflict.^{10–11}

Although we did not assess the synovial presence of deiminated fibrinogen or vimentin in the present study or the presence of intracellular citrullinated proteins in multiple joints of a single patient, we explored these alternative hypotheses indirectly by investigating the relation between PADI4 haplotypes and ACPA independently of the presence of citrullinated antigens. In contrast to the report of Suzuki *et al.*,⁵ we were unable to show an increase of ACPA in patients homozygous for PADI4 haplotype 2. The smaller size of the present study ($n = 59$) compared with the Japanese study ($n = 123$) could be part of the explanation, although the size of the present study allowed us to detect significant differences in ACPA titres with a power of over 80%. Even though the number of haplotype 2 homozygotes was rather small, there was not even a trend towards difference in our study. Although we cannot formally exclude the possibility that larger groups will allow the detection of small differences in ACPA titres between haplotype 1 and 2, this seems unlikely when one considers the large variability in ACPA titres in both groups. Moreover, the stronger association of ACPA levels with synovial intracellular citrullinated proteins on the one hand and with HLA-DR SE on the other raises further questions on the strength and biological relevance of a potential association with PADI4 haplotypes in larger cohorts than in the present study. Finally, it should

be noted that a recent study in a French population was also unable to show a relation between PADI4 haplotypes and serum ACPA levels as determined by both anti-CCP and anti-human deiminated fibrinogen ELISA.¹⁸

An alternative explanation for the discrepancy between the data of Suzuki *et al* and the present study is that the association described by Suzuki may be relatively weak. The association was found only when comparing haplotype 2 homozygotes with the other groups in a dichotomous way, using a test with relatively low specificity (ELISA with citrullinated filaggrin as substrate used at a specificity level of 83.2%, versus a specificity level of 98.5% for the present study). In contrast, they found no difference between haplotype 1 and 2, there was no continuous trend from haplotype 2 homozygotes over heterozygotes to haplotype 1 homozygotes, and the results could not be reproduced using the highly specific anti-CCP2 ELISA.

Finally, it should be noted that the disease association between the functional haplotypes of PADI4 and rheumatoid arthritis was not analysed in this study but recently could not be confirmed in a UK population, although the haplotype frequencies were similar and thus support the assumption that the SNPs analysed represent the same haplotypes as in the Japanese study.⁹ Accordingly, no single PADI4 SNP or haplotype was associated with rheumatoid arthritis in the previously mentioned family based study in a French population.¹⁸ Although neither study undertook full haplotyping, the fact that the haplotypes analysed are largely similar to those described in the Japanese population was recently confirmed in an extensive PADI4 SNP and haplotype analysis in healthy white individuals.¹⁹ However, the latter study also described novel SNPs and haplotypes compared with the Japanese study and thereby emphasised the need for further full haplotyping studies on PADI4 disease association in large white cohorts.¹⁹

Conclusion

While the link demonstrated between synovial intracellular citrullinated proteins and ACPA emphasises the role of deimination of synovial proteins in the pathogenesis of rheumatoid arthritis, our data raise questions about the biological relevance of the described PADI4 haplotypes for this autoimmune process, at least in a European population.

ACKNOWLEDGEMENTS

Tinke Cantaert was supported by a research grant from the Bijzonder Onderzoeksfonds, Ghent University (B/04608). Leen De Rycke was supported by a grant from the Vlaams instituut voor de bevordering van het wetenschappelijk-technologisch onderzoek in de industrie (IWT/SB/11127). Dominique Baeten is a senior clinical investigator of the Fund for Scientific Research–Flanders (FWO Vlaanderen).

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