How is autoimmunity against citrullinated proteins regulated?
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ACPA levels are not associated with PADI 4 haplotypes.

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Abstract

Objectives

Haplotypes of PADI4, encoding for a citrullinating enzyme, were shown to be associated with rheumatoid arthritis (RA) in a Japanese population and were suggested to be related to the presence of anti-citrullinated protein antibodies (ACPA). In order to assess the biological relevance of these PADI4 polymorphisms for RA pathogenesis, the present study explored the relationship between PADI4 haplotypes, the presence of the highly RA-specific intracellular citrullinated proteins in the synovial membrane, and the serum ACPA titers.

Patients and methods

Synovial biopsies and peripheral blood samples were obtained in 59 RA patients. Synovial intracellular citrullinated proteins were detected by immunohistochemistry. Serum ACPA titers were measured with the anti-CCP2 ELISA. PADI4 haplotypes were determined by direct sequencing of the 4 exonic PADI4 single nucleotide polymorphisms.

Results

PADI4 haplotype frequencies as well as 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 significantly higher serum ACPA levels. Similarly, there was no significant association between PADI4 haplotypes and serum ACPA, neither by a continuous analysis using the titers nor by a dichotomous analysis using the diagnostic cut-off. Further analyses in function of homozygotes for haplotype 1, homozygotes for haplotype 2, and heterozygotes (1/2) also failed to demonstrate any significant association between PADI4 polymorphisms and ACPA. This contrasted with the clear association between ACPA levels and HLA-DR shared-epitope.
Conclusion

Whereas the demonstrated link between synovial intracellular citrullinated proteins and ACPA emphasizes the role of deimination of synovial proteins in RA pathogenesis, our data question the biological relevance of the described PADI4 haplotypes for this autoimmune process, at least in a European population.
Introduction

Anti-citrullinated protein antibodies (ACPA) have been extensively documented over the last years as highly specific serological markers for rheumatoid arthritis (RA), resulting in important clinical applications with regard to diagnosis and prognosis (1). Although the pathophysiology of ACPA induction and the role of these antibodies in the pathogenesis of RA remains to be further elucidated, it has convincingly been demonstrated that posttranslational 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 subtype 2 and 4 have been demonstrated in human synovial tissue, the primary disease target of RA (4). In this context, the recent description of an association between rheumatoid arthritis (RA) and a functional haplotype of PADI4, the gene encoding for PAD type 4, is of major interest (5). Indeed, Suzuki et al identified 17 SNPs in the PADI4 gene, of which 8 were associated with RA 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 RA group whereas the other (haplotype 1) was overrepresented in the control group. They indicated that RA patients homozygous for haplotype 2 were more frequently 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 RA-specific presence of intracellular cirullinated proteins in synovium and the colocalization of these proteins with ACPA reactivity, suggesting a role for these proteins in the humoral autoimmune process (6). In order to assess the biological relevance of PADI4 polymorphisms in this process, the present study investigated directly the link between the described PADI4 haplotypes, RA-specific synovial intracellular citrullinated proteins, and ACPA.
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Materials and methods

Patients

The present study included 59 RA patients fulfilling the ACR criteria (7). Fifty nine % were female, with a mean age of 55.5 ± 15.4 (mean ± standard deviation). All patients had active disease with a mean serum C-reactive protein level of 5.2 ± 5.4 mg/L, a mean erythrocyte sedimentation rate of 42.6 ± 24.9 mm/h, and a 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 HLA-DR shared epitope in 83.1% of the patients. Current treatment included disease-modifying antirheumatic drugs in 56.9%, corticosteroids in 43.5%, and non-steroidal anti-inflammatory drugs in 86% of the patients. All patients gave written informed consent to participate to the study as approved by the Ethics Committee of the 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, 8 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 (4 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 2 out 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 titers were measured by the anti-CCP2 ELISA containing synthetic citrullinated peptides as substrate (Immunoscan RA, mark 2, Eurodiagnostica, Arnhem, The 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 98.5% specificity level (8).
PADI4 genotyping

DNA extraction was performed using the genomic DNA purification kit (Puregene, Gentra, Minneapolis, 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). These SNPs correspond also to those used by Barton et al to define haplotype 1 and 2 in a Caucasian UK population (9). Briefly, polymerase chain reaction was performed using the following primersets (Invitrogen, Carlsbad, USA) 5’-TTGTCCACAGCTCTGCC-3’ and 5’-ACACTGCAACCCCCCACAG-3’ for padi4_89 and padi4_90; 5’-GTTCAGATTTCTCATACGGACC-3’ and 5’-GGGATGAGACGGCACTC-3’ for padi4_92 and 5’-GACCTGCCATTGAGGCCAG-3’ 5’-GAATACGATTGGACAGGAGCCAGC-3’ for padi4_104. Sequencing reactions were performed using the Bigdyte Terminator v3.1 Sequencing Reagents and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, 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) as instructed by the manufacturer. The following HLA-DR alleles were considered having the SE: HLA-DRB1*0101, 0401, 0404, and 1001. HLA-DRB1*0405, 0408, and 1402 were not found in our population.

Statistics

A χ²-test was used for comparisons between two discrete variables. Comparison of serum ACPA levels (not-normally distributed, median values and 25-75 percentile are given) was assessed by the Mann-Whitney U test. A p-value of less than 0,05 was considered statistically 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 occurring haplotypes of PADI4, as described by Suzuki et al (5). Two of these exonic SNPs, padi4_92 and padi4_104, were associated with RA 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 patient 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 the frequencies reported by Suzuki et al. in a Japanese population (5) and by Barton et al. in a United Kingdom population (9). This finding supports the assumption that the haplotypes defined by us and by Barton et al (9) by analyzing only the 4 exonic SNPs correspond to those described by Suzuki in a different population (5). However, this can not formally be demonstrated without full haplotype analysis allowing to distinguish more precisely haplotype 1 and 2 from other haplotypes occurring at low frequencies. The remainder of the study will focus on haplotype 1 (n=60) and haplotype 2 (n=40), which are defined similarly in the 3 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 RA synovial tissue samples. Although it is clear that not all synovial citrullinated proteins are RA specific (10,11), we and others indicated previously that the synovial intracellular citrullinated targets recognized by the Biogenesis antibody are RA specific (10-14). Furthermore, the colocalization with ACPA reactivity but not with iNOS (6) 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 titers were significantly higher in the synovial intracellular citrullinated protein positive group (median 982 [303-1800]U/ml) versus the intracellular citrullinated protein negative group (median 343 [4-963] U/ml)(p=0.007), emphasizing their relevance as antigenic targets for ACPA. Therefore, we next analyzed if PADI4 haplotypes were associated
ACPA levels are not associated with PADI 4 haplotypes

PADI4 haplotypes are not associated with ACPA

Since it can however not be excluded 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 titers, irrespective of the presence or absence of specific citrullinated antigens in the synovial membrane. There was no difference in ACPA titers between haplotype 1 (median 750 [140-1149] U/ml) and haplotype 2 (629 [102-1000] U/ml)(p=0.624)(Figure 1E). Similarly, serum ACPA levels were not significantly different between haplotype 1 homozygotes (973 [133-1144] U/ml), heterozygotes (487 [207-966] U/ml), and haplotype 2 homozygotes (629 [7-1018] U/ml)(Figure 1G).

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

Table 1: PADI4 haplotype frequencies in a cohort of 58 rheumatoid arthritis patients, as determined by the 4 exonic SNPs of PADI4. NA = not applicable.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Haplotype frequency</th>
<th>SNP</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>89</td>
</tr>
<tr>
<td>Haplotype 1</td>
<td>51.7% (60/116)</td>
<td>A</td>
</tr>
<tr>
<td>Haplotype 2</td>
<td>34.5% (40/116)</td>
<td>G</td>
</tr>
<tr>
<td>Haplotype 4</td>
<td>9.5% (11/116)</td>
<td>G</td>
</tr>
<tr>
<td>Others</td>
<td>4.3% (5/116)</td>
<td>NA</td>
</tr>
</tbody>
</table>

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 the samples from the PADI4 haplotype 2 group (Figure 1A). Moreover, there were no significant differences between haplotype 1 homozygotes, heterozygotes, and haplotype 2 homozygotes (50.0%, 41.7%, and 25.0%, respectively)(Figure 1C). Thus, the presence of RA-specific intracellular citrullinated proteins in the synovial membrane, which was significantly associated with ACPA titers, was not related to the 2 main PADI4 haplotypes.
Figure 1: Analyzing 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 anti-citrullinated protein antibodies (ACPA), and serum ACPA levels (panel A, C, E, and G). For comparison, a similar analysis was performed for the HLA-DR shared epitope (SE) (panel B, D, F, and H). Positivity (% of samples) for synovial intracellular citrullinated proteins and ACPA are shown in function of haplotypes (panel A and B) and genotypes (panel C and D). Similarly, ACPA levels (units/ml as determined by the anti-CCP2 ELISA; results are represented as median and 25-75 percentile) are shown in function of haplotypes (panel E and F) and genotypes (panel G and H). *p<0.05.
ACPA levels are not associated with PADI 4 haplotypes

| homozygotes and heterozygotes (84.6% versus 50%, p=0.05) and less ACPA positive samples in the haplotype 2 homozygotes (60% versus 87%, p=0.09).

**HLA-DR SE haplotypes are associated with ACPA**

Since the HLA-DR SE may play a role in ACPA induction (17), we determined for comparison the relationhsip between SE on the one hand and the synovial intracellular citrullinated proteins and ACPA on the other hand. 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%), heterozygotes (42.9%), and SE+ homozygotes (41.7%)(Figure 1B and D).

As to ACPA, a dichotomous analysis using the diagnostic cut-off showed no difference between SE- (73.6%) and SE+ (83.1%) haplotypes but showed a trend toward increased ACPA positivity from SE- homozygotes (66.7%) over heterozygotes (77.1%) to SE+ homozygotes (91.7%)(Figure 1B and D). This was confirmed by the continous analysis of the ACPA levels, which were increased in SE+ (865 [252-2008] U/ml) versus SE- (332 [29-1101] U/ml) haplotypes (p=0.025) as well as in SE+ homozygotes (1517 [681-3810] U/ml) compared to heterozygotes (655 [149-1107] U/ml)(p=0.036) and SE- homozygotes (152 [18-488]) U/ml)(p=0.019)(Figure 1F and H). Since SE is thus significantly associated with ACPA levels and may thus 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 demonstrate any difference in serum ACPA titers (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 RA-specific ACPA (2,3), the association of RA with polymorphisms of PADI4 coding for a citrullinating enzyme is potentialy 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 RA-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 indicate a link between PADI4 haplotypes on the one hand and RA-specific synovial intracellular citrullinated proteins and ACPA on the other hand. With regard to PADI4 and deimination of potential ACPA targets, the present study analyzed synovial intracellular citrullinated proteins rather than other candidate targets since 1/ they are highly specific for RA (6,10-14), 2/ they colocalize with ACPA reactivity (6), and 3/ their presence is associated with significantly higher serum ACPA titers (personal observations and present study). This contrasts with other synovial citrullinated proteins which are not specific for RA and which did not show a correlation with ACPA levels (10,11). Taken together, these data strongly support a role for the RA-specific synovial intracellular citrullinated proteins detected in this study as antigenic targets for the described humoral autoimmune process. Whereas we could not demonstrate 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 due 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 titers. Alternatively, it can not be excluded that other synovial targets of ACPA such as citrullinated fibrinogen or citrullinated vimentin (10,15,16) are associated with PADI4 haplotypes. The previously mentioned fact that neither RA-specificity nor a direct relationship with ACPA titers has yet been demonstrated for these targets does not exclude 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 relationship between PADI4 haplotypes and ACPA independently of the presence of citrullinated antigens. In contrast with the report of Suzuki et al (5), we could not demonstrate an increase of ACPA in patients homozygous for PADI4 haplotype 2. The smaller size of the present study (n=59) compared to the Japanese study (n=123) could be part of the explanation, although the size of the present study allowed the detection of significant differences in ACPA titers with a power of over 80%. Even if especially the number of haplotype 2 homozygotes was small, there was not even a trend towards difference in our study. Although it can not be formally excluded that larger groups will allow the detection of small differences in ACPA titers between haplotype 1 and 2, this seems unlikely when one considers the large variability in ACPA titers 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 hand further questions the strenght
ACPA levels are not associated with PADI 4 haplotypes

and biological relevance of a potential association with PADI4 haplotypes in larger cohorts rather than the size of the present study. Finally, it should be noted that a recent study in a French population was also unable to demonstrate a relationship 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 only found when comparing haplotype 2 homozygotes versus the other groups in a dichotomous way using a test with relatively low specificity (ELISA test 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 RA was not analyzed in this study but could recently not be confirmed in a UK population, although the haplotype frequencies were similar and thus support the assumption that the analyzed SNPs represent the same haplotypes as in the Japanese study (9). Accordingly, no single PADI4 SNP or haplotype was associated with RA in the previously mentioned family-based study in a French population (18). Although both studies did not perform a full haplotyping, the fact that the analyzed haplotypes are largely similar to those described in the Japanese population was recently confirmed in an extensive PADI4 SNP and haplotype analysis in healthy Caucasian individuals (19). However, the latter study also described novel SNPs and haplotypes compared to the Japanese study and thereby emphasized the need for further full haplotyping studies on PADI4 disease association in large Caucasian cohorts (19).

In conclusion, whereas the demonstrated link between synovial intracellular citrullinated proteins and ACPA emphasizes the role of deimination of synovial proteins in RA pathogenesis, our data question the biological relevance of the described PADI4 haplotypes for this autoimmune process, at least in a European population.
CHAPTER 5

References


