Investigation of polymorphisms in the PADI4 gene in determining severity of inflammatory polyarthritis

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Background: A functional haplotype of the peptidylarginine deiminase 4 (PADI4) gene has recently been identified as a rheumatoid arthritis susceptibility gene in a Japanese but not in a UK population. One possible explanation for this disparity is that the gene determines severity rather than susceptibility to inflammatory polyarthritis (IP) and that the UK and Japanese cohorts differed in terms of outcome.

Aim: To examine the association between individual PADI4 single nucleotide polymorphisms (SNPs) and haplotypes, with the development and severity of erosions by five years in patients with IP.

Methods: 438 patients from the NOAR inception cohort of patients with IP were x rayed five years after presentation with early IP. Association with four exonic SNPs (padi4_89*G/A, padi4_90*T/C, padi4_92*G/C, and padi4_104*T/C), mapping to the PADI4 gene and defining a haplotype previously reported to be associated with rheumatoid arthritis, was investigated. Patients were compared for the presence, extent, and progression of erosions by five years and the presence of antibodies to citrullinated peptide (anti-CCP antibodies).

Results: There was no association between individual PADI4 SNPs or haplotypes and the development or extent of erosions by five years. Restricting analysis to patients who satisfied ACR criteria for rheumatoid arthritis by five years did not alter the conclusions. No association with presence of anti-CCP antibodies was detected.

Conclusions: No evidence was found for association of the PADI4 gene with severity as assessed by erosive outcome at five years or with presence of anti-CCP antibodies in patients with IP.

Recently, an association of rheumatoid arthritis with the peptidylarginine deiminase 4 (PADI4) gene (NM_012387) has been described in a Japanese population. The gene encodes a member of a family of enzymes that post-translationally convert arginine residues to citrulline. It had previously been noted that anticyclic citrullinated peptide (anti-CCP) antibodies appear to be specific for rheumatoid arthritis, predict the development of the disease in patients with undifferentiated inflammatory arthritis, and arise early in the disease course, suggesting that they may have a pathogenic role. A susceptibility haplotype of the PADI4 gene was shown to affect stability of mRNA transcripts and was associated with levels of antibody to anti-CCP antibodies in the sera of Japanese patients with rheumatoid arthritis. Hence it was postulated that the presence of the susceptibility haplotype results in greater transcript stability, in turn leading to more citrullination of epitopes, which then become targets for anti-CCP antibodies. However, in previous work, neither we nor others have shown association of this susceptibility haplotype in white European rheumatoid arthritis populations.

One explanation could be that the Japanese and European cohorts differed in terms of severity. In support of this, the presence of anti-CCP antibodies has previously been shown to predict erosive outcome in patients with early inflammatory polyarthritis (IP), suggesting that they may be severity rather than susceptibility markers and, likewise, polymorphism within the gene may be associated with severity rather than susceptibility. In order to separate these effects, it is necessary to investigate an inception cohort of patients all followed prospectively to quantify disease outcome. The Norfolk Arthritis Register (NOAR) provides such a resource. It aims to collect information on all incident cases of inflammatory polyarthritis (IP) (defined as swelling of two or more joints lasting for four or more weeks, after exclusion of other causes) presenting to a primary care physician in a geographically defined region, and is a true primary care based inception cohort. Data collection is not restricted to patients fulfilling ACR criteria for rheumatoid arthritis at presentation because the proportion of patients satisfying the criteria changes over time. It is therefore preferable, when studying early disease, to study the whole population of patients with IP and to stratify at different time points for whether or not patients satisfy criteria for rheumatoid arthritis.

The aim of the present study was thus to investigate association of the PADI4 SNPs and haplotypes with the presence and extent of radiographic erosions by five years and with presence of anti-CCP antibodies.

METHODS

Study design

A large prospective inception cohort study was carried out to investigate whether the presence of polymorphisms of the PADI4 gene played a role in determining erosive outcome in patients with IP (of which rheumatoid arthritis is a major subset). Association of four PADI4 gene single nucleotide polymorphisms (SNPs), which together define a functional haplotype previously associated with rheumatoid arthritis in a Japanese population, was tested with the presence and extent of radiological erosions by five years and with the presence of anti-CCP antibodies.

Abbreviations: ACR, American College of Rheumatology; anti-CCP, anticyclic citrullinated peptide antibody; DMARD, disease modifying antirheumatic drug; EM, expectation maximisation; HAQ, health assessment questionnaire; IP, inflammatory polyarthritis; NOAR, Norfolk Arthritis Register; SNP, single nucleotide polymorphism

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Baseline clinical data are recorded and blood taken for research nurse using a standard questionnaire and examination. Baseline radiographic (x-rays) and blood tests are performed. All cases with IP (defined as swelling of two or more joints lasting for four or more weeks) within the region formerly known as the Norwich Health Authority are assessed by a rheumatologist with an interest in rheumatoid arthritis. ACR classification criteria for rheumatoid arthritis are satisfied, using a cumulative evaluation as described previously.

As part of a separate study, the first 380 patients recruited to the NOAR were studied.

Subjects
Patients were recruited from the NOAR. The case ascertainment procedure has been described previously but, briefly, all cases with IP (defined as swelling of two or more joints lasting for four or more weeks) within the region formerly known as the Norwich Health Authority are assessed by a research nurse using a standard questionnaire and examination. Baseline clinical data are recorded and blood taken for RF measurement and DNA extraction. Patients are reviewed annually and at each assessment they are scored as to whether ACR classification criteria for rheumatoid arthritis are satisfied, using a cumulative evaluation as described previously.

All patients had radiographs of the hands and feet five years after registration. Briefly, x-rays of the hands and feet were scored by two observers using the Larsen method. A third observer arbitrated in cases of disagreement. A subset of patients who had three or more ACR criteria had their x-rays at one year for the purposes of this analysis. The first 438 consecutive subjects who were successfully followed up for five years and had provided a blood sample for DNA extraction were studied.

All patients were of UK white (Europid) ethnic origin, provided written informed consent, and were recruited with ethical committee approval (LREC 2003-075).

Genotyping methods
Four PADI4 SNPs (padi4_89G/A, padi4_90T/C, padi4_92G/C (rs874881), and padi4_104T/C (rs1748033)) were genotyped as described previously.

Anti-CCP antibody
As part of a separate study, the first 380 patients recruited to NOAR with x-rays available by two years were tested for the presence of anti-CCP antibody. This was an in house first generation test carried out using an enzyme linked immunosorbent assay (ELISA) (done in collaboration with the Leiden University Medical Centre, Netherlands). Two hundred and fourteen of these patients were included in the current cohort.

Statistical analysis
Patients were defined as having erosive arthritis if they had a Larsen score of ≥2 in any hand or foot joint. Both the presence of any erosion and the actual Larsen score at five years were used as outcome measures to assess the role of the PADI4 polymorphisms and the Japanese susceptibility haplotype. Genotype frequencies of each SNP were compared between cases who developed erosions by one and five years and those without, using Fisher’s exact test. Further analyses were restricted to cases who satisfied ACR criteria for rheumatoid arthritis by five years, or who were rheumatoid factor (RF) positive at baseline. The two sample (Mann–Whitney) test was used to test for an influence of allele frequency on the following disease features: Larsen score at 1 and 5 years; the number of tender and swollen joints at baseline and 5 years; and the health assessment questionnaire (HAQ) score at baseline and five years. Subgroup analysis was also undertaken restricting analysis to those who satisfied ACR criteria for rheumatoid arthritis by five years.

As mentioned above, in the Japanese population, a haplotype defined by the four SNPs genotyped in the current study was found to affect mRNA stability. The analysis package SNPHAP (http://www-gene.cimr.cam.ac.uk/clayton/software/) was therefore used to estimate and assign haplotypes. This program uses the expectation maximisation (EM) algorithm and the software assigns haplotypes to...
individuals, provides information about the probability of that assignment, and also allows some missing data—for example, as a result of PCR failure. Two haplotypes are assigned to each individual and individuals in whom haplotypes were assigned with a probability of less than 90% were excluded from further analysis in order to ensure stringency. The “susceptibility” haplotype, as defined in the Japanese population, was referred to as 2 and all other haplotypes as 1.

As the Larsen score is a count, negative binomial regression was used to model the influence of the Japanese susceptibility PADI4 haplotype on Larsen score at one and five years. Negative binomial regression was used rather than Poisson regression, as the scores obtained display greater variation than a Poisson distribution. Hence Poisson regression underestimates the standard errors. The outcome from the negative binomial regression is a multiplier for each group—for example, a coefficient of 1.2 implies that the Larsen score in that group is, on average, 20% higher than the referent group.

Finally, as treatment with disease modifying antirheumatic drugs (DMARDs) may mask genetic associations, all analyses were carried out after adjustment for ever using a DMARD.

**Power**

Sample sizes were calculated based on published allele frequencies (minor allele frequencies of 33–40%) so that each of the association studies had 80% power to detect a genotype relative risk of 2.0 (the susceptibility effect reported for the padi4_104*T/C SNP in the Japanese population) at the 5% significance level for development of erosions by five years.

**Correction for multiple testing**

Association with four SNPs and one haplotype were investigated in this study and, in addition, the data were stratified as described above to fully explore whether the PADI4 gene is associated with severity of IP. As the SNPs are tightly linked, it is difficult to determine accurately the Bonferroni correction that should be applied, as the test assumes independence of loci. In this study, we used a correction factor of 20. The uncorrected probability (p) values are presented, as well as the corrected p values where the uncorrected value achieved statistical significance.

### RESULTS

#### Patients

In all there were 438 IP cases with x ray data at five years. Their mean (SD) age was 53.3 (14.3) years; 286 (65.3%) were...
female; 114 (25.8%) were RF positive at baseline and 166 (37.9%) by five years; and 197 (45.0%) satisfied ACR criteria for rheumatoid arthritis at baseline and 317 (72.4%) by five years. Carriage of shared epitope alleles was as follows: 39.5% for rheumatoid arthritis at baseline and 317 (72.4%) by five years; and 197 (45.0%) satisfied ACR criteria for rheumatoid arthritis by five years did not alter the findings substantially. The primary outcome measure was the development of erosions; this is accepted as an objective and reliable outcome measure and the presence of erosions is used in many studies as a surrogate marker for quantifying disease severity. However, as erosions are part of the criteria for rheumatoid arthritis, studying prevalent cases with variable disease duration would have made separation of susceptibility from severity factors difficult. Therefore, by investigating association in an inception cohort of patients who were all followed prospectively and were individually but not by haplotype (uncorrected p value = 0.10) (table 2). It should be noted that these associations did not remain significant after a Bonferroni correction was applied.

By contrast with a previous report in a Japanese population, the PADI4 SNPs and “susceptibility” haplotype were not associated with presence of anti-CCP antibodies at baseline in this UK cohort of IP patients (table 4). Similarly, no association with presence of RF at baseline or satisfaction of ACR criteria for rheumatoid arthritis by five years was detected (table 4).

### DISCUSSION

This is the first study to examine in detail the possible link between polymorphism in the PADI4 gene and disease severity in IP and rheumatoid arthritis. No association of PADI4 polymorphism with severity was observed. This was the case whether measures of disease severity or activity at either baseline or by five years were considered.

One of the strengths of the current study is that analysis was not restricted to ACR defined rheumatoid arthritis cases with established disease, because the ACR criteria perform poorly in early disease and it is in early disease that the identification of genes that determine prognosis is likely to have the greatest impact by allowing better targeting of aggressive treatments. However, the majority (75%) of these patients subsequently satisfied ACR criteria for rheumatoid arthritis by five years, and restricting analysis to patients satisfying ACR criteria for rheumatoid arthritis by five years did not alter the findings substantially. The primary outcome measure was the development of erosions; this is accepted as an objective and reliable outcome measure and the presence of erosions is used in many studies as a surrogate marker for quantifying disease severity. However, as erosions are part of the criteria for rheumatoid arthritis, studying prevalent cases with variable disease duration would have made separation of susceptibility from severity factors difficult. Therefore, by investigating association in an inception cohort of patients who were all followed prospectively and were

### Table 4

<table>
<thead>
<tr>
<th>SNP allele</th>
<th>RA by 5 years (%)</th>
<th>Non-RA by 5 years (%)</th>
<th>RF+ at baseline (%)</th>
<th>RF– at baseline (%)</th>
<th>CCP+ at baseline (%)</th>
<th>CCP– at baseline (%)</th>
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<tbody>
<tr>
<td>Padi4_89</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>G</td>
<td>370 (59.3)</td>
<td>129 (60.8)</td>
<td>124 (59.0)</td>
<td>373 (60.0)</td>
<td>44 (56.4)</td>
<td>216 (65.9)</td>
</tr>
<tr>
<td>A</td>
<td>254 (40.7)</td>
<td>83 (39.2)</td>
<td>86 (41.0)</td>
<td>249 (40.0)</td>
<td>34 (43.6)</td>
<td>112 (34.1)</td>
</tr>
<tr>
<td>Comparison</td>
<td>p = 0.75</td>
<td>p = 0.87</td>
<td>p = 0.87</td>
<td>p = 0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Padi4_90</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>354 (58.4)</td>
<td>120 (58.3)</td>
<td>116 (58.6)</td>
<td>355 (58.8)</td>
<td>45 (56.3)</td>
<td>203 (63.8)</td>
</tr>
<tr>
<td>C</td>
<td>252 (41.6)</td>
<td>86 (41.7)</td>
<td>82 (41.4)</td>
<td>249 (41.2)</td>
<td>35 (43.7)</td>
<td>115 (36.2)</td>
</tr>
<tr>
<td>Comparison</td>
<td>p = 1.0</td>
<td>p = 0.74</td>
<td>p = 0.74</td>
<td>p = 0.25</td>
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<td>Padi4_92</td>
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<tr>
<td>G</td>
<td>348 (55.9)</td>
<td>116 (56.3)</td>
<td>116 (56.3)</td>
<td>347 (56.3)</td>
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<tr>
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<td>90 (43.7)</td>
<td>90 (43.7)</td>
<td>269 (43.7)</td>
<td>34 (44.7)</td>
<td>125 (38.3)</td>
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<tr>
<td>Comparison</td>
<td>p = 0.94</td>
<td>p = 1.0</td>
<td>p = 1.0</td>
<td>p = 0.36</td>
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<tr>
<td>T</td>
<td>425 (67.0)</td>
<td>148 (70.5)</td>
<td>133 (63.9)</td>
<td>435 (68.8)</td>
<td>50 (62.5)</td>
<td>234 (72.2)</td>
</tr>
<tr>
<td>C</td>
<td>209 (33.0)</td>
<td>62 (29.5)</td>
<td>75 (36.1)</td>
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<td>30 (37.5)</td>
<td>90 (27.8)</td>
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<td>Comparison</td>
<td>p = 0.39</td>
<td>p = 0.20</td>
<td>p = 0.20</td>
<td>p = 0.10</td>
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<tr>
<td>Haplotype*</td>
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<tr>
<td>1</td>
<td>413 (67.0)</td>
<td>146 (70.9)</td>
<td>129 (63.9)</td>
<td>425 (69.0)</td>
<td>49 (62.8)</td>
<td>233 (72.4)</td>
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<td>2</td>
<td>203 (33.0)</td>
<td>60 (29.1)</td>
<td>73 (36.1)</td>
<td>191 (31.0)</td>
<td>29 (37.2)</td>
<td>89 (27.6)</td>
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<tr>
<td>Comparison</td>
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<td>p = 0.19</td>
<td>p = 0.19</td>
<td>p = 0.10</td>
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</tr>
</tbody>
</table>

*The PADI4 susceptibility haplotype, as defined in the Japanese population, was referred to as 2 and all other haplotypes as 1. CCP, cyclic citrullinated peptide; RA, rheumatoid arthritis; RF, rheumatoid factor; SNP, single nucleotide polymorphism.
unselected for the presence of erosions at study entry, any bias which might have arisen from this cause should have been eliminated.

The effects of treatment on disease severity may obscure the investigation of genetic factors. Patients with severe disease at baseline who receive treatment early are expected to have milder disease over time than if they had remained untreated. We have tried to account for this possible confounding by treatment allocation using an adjustment in the analysis (whether patients had ever received a DMARD) but this adjustment may be imperfect.

Although the study was adequately powered to detect an associated SNP or haplotype conferring an odds ratio of 2.0 for the development of erosions by five years, if the effect size is small much larger sample sizes will be required. It should be noted, however, that previously identified predictors of erosions—such as carriage of shared epitope alleles, presence of RF at baseline, and presence of anti-CCP antibodies at baseline—were all strongly predictive of erosions by five years in this IP cohort (p = 2.6 × 10^{-6}, 3.0 × 10^{-8}, and p = 7.4 × 10^{-5}, respectively).8 15 16

A previous study in a Japanese population found that the haplotype was associated with presence of anti-CCP antibodies, although data were only available for 123 patients.1 In our study, anti-CCP antibody data were available for 214 patients. We found no association of PADI4 SNPs or haplotype with the presence of anti-CCP antibodies, although there was a trend towards association for the haplotype (odds ratio = 1.5 (95% confidence interval, 0.9 to 2.07), p = 0.10). In order to confirm an association of the haplotype with anti-CCP antibodies, a much larger sample size would be required. In both the Japanese study and our own, haplotypes were estimated using the EM algorithm. Previous work has shown very similar frequencies for estimated and directly sequenced haplotypes in the white population.12

The results presented herein suggest that polymorphism within the PADI4 gene itself does not play a major role in determining erosive or severe outcome in IP in the United Kingdom population. Hence genotyping patients for these polymorphisms at presentation will not help in targeting treatments to patients likely to develop more severe disease.

ACKNOWLEDGEMENTS

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Notes