An association between the CTLA4 exon 1 polymorphism and early rheumatoid arthritis with autoimmune endocrinopathies

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Abstract

Objective. To examine the allelic association of the single nucleotide polymorphism (CTLA4A/G) in exon 1 of the cytotoxic T lymphocyte antigen-4 (CTLA4) gene with early rheumatoid arthritis (RA).

Methods. One hundred and twenty-three unrelated white probands with early RA from the north-east of England and 349 local ethnically matched controls were studied. The CTLA4A/G polymorphism was genotyped with a polymerase chain reaction (PCR) method and digestion with the restriction enzyme Bst71I. Probands were also screened by allele-specific PCR for alleles HLA DRB1*01 and DRB1*04.

Results. The frequency of the G allele at CTLA4A/G was significantly increased in probands with early RA compared with controls (43 vs 36%; P = 0.028, odds ratio (OR) 1.35, 95% confidence interval (CI) 1.01–1.82). Most of this increased frequency was attributable to RA individuals with coexisting autoimmune thyroid disease or type 1 diabetes (58 vs 36% in controls; P = 0.005, OR 2.50, CI 1.29–4.84). The frequency of the G allele in RA patients without autoimmune endocrinopathy was 40%, which was not significantly different from that in controls (P = 0.140).

Conclusion. The association between the CTLA4 G allele and early RA is largely explained by individuals with RA who have coexisting autoimmune endocrinopathies.

Key words: CTLA4, Rheumatoid arthritis, Type 1 diabetes, Autoimmune thyroid disorder.
AITD [7–11]. Although previous case-control studies in different populations have suggested a possible association of CTLA4 alleles with RA, the results of these studies are not conclusive and are sometimes contradictory [12–15]. In the present study, we demonstrate an association between the G allele of the CTLA4 exon 1 polymorphism (CTLA4A/G) and RA, which is explained largely by the presence of coexisting autoimmune endocrinopathies.

Patients and methods

Patients

Consecutive patients referred to our rheumatology outpatient service with inflammatory arthritis were screened to identify individuals who fulfilled the 1987 modified criteria for RA [16] and in whom the interval from the onset of persistent symptoms was less than 2 yr. One hundred and twenty-three white patients were recruited. The Westergren erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) concentration and rheumatoid factor were analysed at presentation. Rheumatoid factor was analysed with a particle agglutination kit (Fujirebio, Tokyo, Japan), and final dilution titres of 1:40 and above were interpreted as positive. Radiographs of the hands and feet were taken at presentation and were read by an independent radiologist to determine the presence or absence of erosions. We also studied 349 local white subjects without clinical evidence or a family history of autoimmune disease as controls. The majority of these subjects were recruited from the general population from the patient register of the Newcastle Family Health Services Authority, and 92 were general out-patients with non-autoimmune disorders. Studies were carried out with the approval of the district ethics committee.

CTLA4 genotyping

Genomic DNA was obtained from venous blood from each subject using the BACCII kit (Nucleon Biosciences, Glasgow, UK). The CTLA4A/G polymorphism, which encodes a threonine (GCC) to alanine (ACC) substitution at codon 17 in exon 1 of the CTLA4 gene, was amplified by the polymerase chain reaction (PCR) from genomic DNA followed by digestion with the restriction enzyme Bst71I (Promega, Southampton, UK), as described previously [9]. The primers were 5′-CCA CGG CTT CCT TTC TCG TA3′ (forward) and 5′-AGT CTC ACT CAC CTT TGC AG-3′ (reverse). The restriction enzyme Bst71I cuts the 328 base pair (bp) PCR product only if the G allele is present at this site, resulting in fragments of 244 and 84 bp, which were resolved on a 2.5% agarose gel.

HLA DRB1*01 and DRB1*04 typing

Typing for the HLA DRB1*01 (DR1) and HLA DRB1*04 (DR4) alleles was performed in our laboratory using methods, primers and controls obtained from the Tissue Typing Laboratory at the Northern Regional Blood Transfusion Centre, Barrack Road, Newcastle-upon-Tyne. The method involved PCR with sequence-specific oligonucleotide primers. HLA DRB1*01 was genotyped with the primers 5′-TTG TGG CAG CTT AAG TTG GTA T-3′ and 5′-CCG CCT CTC CAG GAG-3′. These identify the DRB1* alleles 0101, 01021, 01022 and 0104. Similarly, HLA DRB1*04 (DR4) was genotyped with the primers 5′-GTT TCT TGG AGC AGG TTA AAC-3′, 5′-CTG CAC TGT GAA GCT CTC AC-3′ and 5′-CTG CAC TG CAG GCT CTC CA-3′. These identify the DRB1* alleles 04011–0407, 0410–0412, 0415–0417, 0421, 0422, 0424, 0425, 1122, 1410, 0419, 0420 and 0423.

Statistical analysis

Comparisons of the allele frequencies at the CTLA4A/G polymorphism between patient and control groups were performed using Fisher’s exact test on 2×2 contingency tables. Odds ratios (OR) and 95% confidence intervals (CI) were calculated with Woolf’s method. The relationships of CTLA4A/G genotype with ESR and CRP level at presentation were analysed with the Mann–Whitney U-test. GraphPad Prism version 2.01 was used to compute these statistical analyses.

Results

We genotyped 123 subjects with early RA and 349 controls for the CTLA4A/G polymorphism. The demographic and clinical characteristics of the early RA patients are shown in Table 1. Nineteen (15%) of these patients had a history of coexistent autoimmune endocrinopathies (AITD or IDDM).

We found a significantly increased frequency of the G allele of the CTLA4A/G polymorphism in early RA subjects compared with controls (P = 0.028 vs controls, OR 1.35, 95% CI 1.01–1.82) (Table 2). In the 19 RA subjects with coexisting autoimmune endocrinopathy (AITD or IDDM), there was a strong association with the CTLA4 G allele (58 vs 36% in controls, P = 0.005, OR 2.50, 95% CI 1.29–4.84) (Table 3). In a smaller subgroup of 13 patients with endocrinopathy, who were also DR1- or DR4-positive, the G allele frequency was 65 vs 36% in controls (P = 0.002, OR 3.43, 95% CI 1.51–7.81). The prevalence of the CTLA4 G allele amongst the RA subjects with autoimmune endocrinopathy was significantly different from that of RA subjects without endocrinopathy (58 vs 40%; P = 0.030,

| Table 1. Demographic and clinical characteristics of the early RA cohort (n = 123) |
|---------------------------------------------------------------|------------------|
| Females                                                       | 80 (65%)         |
| Mean age (yr)                                                 | 53.7 (range 18–85) |
| Mean disease duration (days)                                  | 285 (range 42–730) |
| Positive for rheumatoid factor                                | 87 (71%)         |
| Coexistent AITD                                               | 15 (12%)         |
| Coexistent IDDM                                               | 4 (3%)           |
| Radiographic erosive changes at presentation                  | 45 (36.5%)       |
| Mean ESR: mm/h (s.d.)                                         | 28.5 (21.7)      |
OR 2.07, 95% CI 1.03–4.18). The *CTLA4* G allele frequency of RA subjects without endocrinopathy was not significantly different from that of controls (40 vs 36%, \(P = 0.140\)).

In the patients with radiological evidence of erosions at presentation, the G allele frequency was 46% compared with 41% \( (P = 0.28) \) in those who had normal X-rays. There was no significant difference in the G allele frequency between *DR1*/*DR4*-positive and *DR1*/*DR4*-negative RA subjects (Table 3). No significant differences were found in the G allele frequency between male and female patients (50 vs 39%, \( P = 0.11) \), or between patients positive or negative for rheumatoid factor (47 vs 41%, \( P = 0.216) \). No association was found between different genotypes of the *CTLA4* polymorphism and ESR or CRP values measured at the time of presentation (data not shown).

### Discussion

Our study shows that the G allele of the *CTLA4A/G* polymorphism is more prevalent in early RA patients than in controls \( (P = 0.028) \). However, this finding is explained largely by the strong association of the G allele with autoimmune endocrinopathies in our cohort (Table 3). In contrast to the four previous studies of the *CTLA4A/G* polymorphism in RA \cite{12–15}, the present study took account of the autoimmune endocrinopathies, which are known to be independently associated with *CTLA4* alleles \cite{7–11}. The association of RA with *CTLA4* alleles reported in other RA cohorts may also be due to cases of coexisting AITD or IDDM \cite{12–14}. Seidl et al. \cite{12} reported a significant increase in the G allele frequency in German RA patients who were positive for the *HLA-DRB1*/*0401* allele \cite{12}. In a similar Spanish study, a significant increase in A/G heterozygosity for the *CTLA4A/G* polymorphism was found only in *HLA-DR3*-positive subjects, particularly in *DR3*-positive women \cite{13}. These reports of apparent *CTLA4* association within HLA subgroups of RA patients could also be explained by the known associations of *HLA DR3* and *DR4* alleles with AITD and IDDM respectively.

There are other possible reasons for the discrepancies observed between our study and previous *CTLA4* association studies \cite{12–15}. Case–control studies are very sensitive to the selection of an appropriate control population. In the study of Barton et al. \cite{15}, which found no association between *CTLA4* alleles and RA, the G allele frequency in the UK controls (ethnicity not detailed) is given as 46%. This is much higher than the G allele frequencies in the UK white controls seen in our present study (36%) and in another large UK study (32%) \cite{10}. Furthermore, Barton et al. derived their patients from a pan-UK national repository, whilst our patients are from a stable population in a more limited geographical area and are likely to be more genetically homogeneous. Nevertheless, it is interesting to note that the G allele frequency in our RA group (43%) is similar to that found by Barton’s study (42%) \cite{15}. However, the loci for complex multigenic disorders may make different contributions to disease susceptibility in populations of diverse origins. Such genetic heterogeneity in different white populations has been demonstrated previously for *CTLA4* in IDDM and Graves’ disease \cite{8, 9}, and similar heterogeneity may be present in RA.

Though associated with a number of autoimmune diseases, this *CTLA4A/G* polymorphism, which encodes a threonine to alanine change within the signal peptide of CTLA-4, has an unknown function. However,

### Table 2. *CTLA4A/G* allele frequency for subjects with RA and controls

<table>
<thead>
<tr>
<th>Genotype frequencies</th>
<th>RA (n = 123) (n (%)</th>
<th>Controls (n = 349) (n (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>20 (16)</td>
<td>45 (13)</td>
</tr>
<tr>
<td>AG</td>
<td>66 (53)</td>
<td>158 (45)</td>
</tr>
<tr>
<td>AA</td>
<td>38 (31)</td>
<td>146 (42)</td>
</tr>
</tbody>
</table>

**Allele frequencies**

| G        | 105 (43)* | 248 (36) |
| A        | 141 (57)  | 450 (64) |

*\( P = 0.028, \ OR 1.35 \ (95\% \ CI 1.01–1.82) vs \) controls.

### Table 3. *CTLA4A/G* allele frequency in RA subgroups

<table>
<thead>
<tr>
<th>Genotype frequencies</th>
<th>RA with erosion (n = 45) (n (%))</th>
<th>RA, no erosion (n = 78) (n (%))</th>
<th>RA with AITD/IDDM (n = 19) (n (%))</th>
<th>RA, no AITD/IDDM (n = 104) (n (%))</th>
<th>RA, <em>DR1/4</em>+ (n = 84) (n (%))</th>
<th>RA, <em>DR1/4</em>− (n = 39) (n (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>9 (20)</td>
<td>11 (14)</td>
<td>6 (32)</td>
<td>14 (13)</td>
<td>14 (17)</td>
<td>6 (15)</td>
</tr>
<tr>
<td>AG</td>
<td>23 (51)</td>
<td>42 (54)</td>
<td>10 (53)</td>
<td>55 (53)</td>
<td>45 (54)</td>
<td>20 (51)</td>
</tr>
<tr>
<td>AA</td>
<td>13 (29)</td>
<td>25 (32)</td>
<td>3 (16)</td>
<td>35 (34)</td>
<td>25 (30)</td>
<td>13 (33)</td>
</tr>
</tbody>
</table>

**Allele frequencies**

| G        | 41 (46)* | 64 (41) |
| A        | 49 (54)  | 92 (59) |

*\( P = 0.042, \ OR 1.52 \ (95\% \ CI 0.97–2.36) vs \) controls; \( P = 0.28, \ OR 1.20 \) vs RA and no erosions.

\( P = 0.005, \ OR 2.50 \ (95\% \ CI 1.29–4.84) vs \) controls; \( P = 0.030, \ OR 2.07 (1.03–4.18) \) vs RA and no AITD or IDDM.

*\( P = 0.035, \ OR 1.39 \ (95\% \ CI 0.99–1.96) vs \) controls; \( P = 0.41, \ OR 1.11 \) vs RA and *DR1/4*−.
it is possible that the polymorphism may subtly affect the membrane localization or folding of the nascent CTLA4 peptide. Further studies are necessary to determine whether \textit{CTLA4}\textsubscript{AAG} is pathogenic or is simply in linkage disequilibrium with a true susceptibility polymorphism.

Despite the lack of significant \textit{CTLA4} allelic association in RA patients without autoimmune endocrinopathies, functional studies support a possible role for the \textit{CTLA4} gene in arthritis [17, 18]. \textit{CTLA4} fused to the Fc fragment of a human IgG (CTLA4Ig) has been shown to block the presentation of arthritogenic antigens to T cells by synovial dendritic cells [17] and to prevent the onset of collagen-induced arthritis in the diabetes-resistant BB\textsubscript{Wor} rat [18]. More studies are warranted to explore the role of \textit{CTLA4} in the pathogenesis of RA.

Acknowledgements

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References