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A single nucleotide polymorphism in exon 1 of cytotoxic T-lymphocyte-associated-4 (CTLA-4) is not associated with rheumatoid arthritis

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Abstract

Background. Rheumatoid arthritis (RA) is an oligogenic disease for which only one susceptibility locus has been identified to date. Genes involved in T-cell regulation are potential candidates. Association to cytotoxic T-lymphocyte-associated-4 (CTLA-4) protein, a negative regulator of T-cell activation, has previously been described in a subset of German RA patients carrying the HLA DRB1*0401 subtype. Linkage and association with another oligogenic autoimmune disease, insulin-dependent diabetes mellitus, has also been described in a Spanish population.

Objective. To investigate the association of CTLA-4 with RA in Spanish and UK subjects.

Methods. Caucasoid UK RA patients ($n = 192$), UK controls ($n = 96$), Spanish RA patients ($n = 136$) and Spanish controls ($n = 144$) were typed for an A/G bi-allelic polymorphism in exon 1 of CTLA-4 using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) (enzyme).

Results. No significant differences in the frequency of the G allele or the GG genotype were found in either the UK or Spanish RA patients compared with controls.

Conclusion. No significant evidence was found of an association between RA and CTLA-4.

KEY WORDS: CTLA-4, SNP, Rheumatoid arthritis, Susceptibility locus, autoimmunity, genetic.

Susceptibility to rheumatoid arthritis (RA) is determined by both genetic and environmental factors. Twin and family studies can be used to estimate the size of the genetic component. The excess concordance in monozygotic (MZ) compared with dizygotic twins (15% compared with 3.6%) [1], when analysed using Falconer's correction, suggests that the genetic contribution to disease development approaches 50–60% [2].

Only one RA susceptibility locus, the major histocompatibility complex (MHC), has been identified to date, although a single-locus model for inheritance of RA has been rejected. HLA has been estimated as accounting for approximately 40% of the genetic component of susceptibility to RA [3]. It is likely that a number of other genes are involved, each contributing a small amount to the total genetic component.

T cells play a major role in the pathogenesis of RA. Based on animal models of inflammatory disease, the success of anti-T-cell therapy in RA and the association with HLA DR4, it has been proposed that RA is a T-cell-mediated autoimmune disease. Regulators of T-cell activity are, therefore, candidates for influencing disease susceptibility. Cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) is a negative regulator of T-cell activation. Mice deficient in CTLA-4 develop a rampant lymphoproliferative disorder resulting in premature death [4]. The *CTLA-4* gene in humans lies on chromosome 2q33 and a microsatellite polymorphism has been identified in the 3′ untranslated region of the gene. This polymorphism has been previously linked to type I insulin-dependent diabetes mellitus (IDDM) in an Italian population but not in a UK population [5]. A bi-allelic G/A polymorphism has also been identified at position 49, exon 1 of the gene [6]. This encodes a Ala/Thr substitution and, although unlikely to be functionally important, may be in linkage disequilibrium with a nearby disease susceptibility gene. The G allele has been associated with IDDM in Spanish, Belgian and Italian populations [5], and with Graves' disease in German [7], US [8] and Hong Kong Chinese [6] patients. Recently, an association with the G allele has been described in a subset of patients with RA in a German population. RA patients carrying the HLA DRB1*0401 subtype were significantly more likely to be homozygous for the GG genotype, although no differences in G allele frequencies were seen in the data set as a whole [9]. Furthermore, an association of the

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G allele with early-onset persistently pauciarticular juvenile chronic arthritis with chronic iridocyclitis has been reported [10].

CTLA-4 is a potential candidate for other T-cell-mediated autoimmune diseases. In this study, we investigated the association of CTLA-4 with RA in UK and Spanish populations.

Materials and methods

Patients and controls

UK samples. DNA was available from 64 unrelated RA cases from the ARC National Twin study and 128 RA probands from the ARC National Repository. All cases satisfied the 1987 modified ARA criteria [11]. Eighty-three per cent were female. The mean age at onset was 40 yr with a range of 16–78 yr. The mean disease duration was 15.6 yr with a range of 0–39 yr. Sixty-nine per cent were seropositive, 47% nodular and 82% erosive. Eighty per cent had received some disease-modifying anti-rheumatic drug (DMARD) treatment and the average was 2.5 DMARDs.

Normal individuals ($n = 96$), with no history of inflammatory joint disease, were recruited from general practice. Fifty-seven per cent were female and the mean age was 48 yr with a range of 19–71 yr.

Spanish samples. DNA was obtained from 136 patients with RA from the Galicia region of northern Spain. These patients were all referred to the Xeral-Calde Hospital by general practitioners or by self-referral to the Accident and Emergency Department. Seventy-one per cent of patients were female. The mean age at disease onset was 49 yr (range 16–78 yr) and the average disease duration was 11.6 yr (0–56 yr). All were seropositive, 81% were erosive and 18% nodular. Only four cases had never received DMARD therapy and the others on average had received 2.7 DMARDs.

Control samples were obtained from the same population ($n = 144$). Forty-nine per cent of controls were female and age ranged from 16 to 90 yr.

Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP)

A G/A polymorphism exists at position 49 in exon 1 of the *CTLA-4* gene [6]. A recognition site for the restriction enzyme *Bbv*I (New England Biolabs, Hertfordshire, UK) is created when the G allele is present but not when the A allele is present. An additional *Bbv*I recognition site upstream of the polymorphism acts as an internal control.

Primers were synthesized with sequences complementary to DNA at position 635 (forward: 5′-GTCAAGGGACCATTGAAG-3′) and 1301 (reverse: 5′-CTTTGCAGAAGACAGGGATGA-3′). DNA containing the polymorphic site was amplified by PCR under the following conditions: 1 min denaturation at 95°C, 1 min annealing at 55°C and 2 min elongation at 72°C for 35 cycles. Restriction enzyme digestion was performed at 37°C overnight and digested samples subjected to electrophoresis in a 2% agarose gel for 30 min at 200 V in Tris–borate electrophoresis buffer. The gels were viewed under a transilluminator.

DNA from 192 Caucasian UK RA patients, 96 healthy UK controls, 136 Spanish RA patients and 144 healthy Spanish controls was analysed for the bi-allelic G/A polymorphism.

Statistical analysis

Allele frequencies in patient and control groups were calculated by direct counting. The association of the G allele with RA was assessed by calculating the odds ratio (OR) and 95% confidence intervals (95% CI) for the G phenotype and GG genotype. The level of significance was determined by χ^2 and Fisher's exact tests. No correction was made for multiple testing. Assuming a dominant mode of inheritance, the study had 80% power to detect a genotypic relative risk (RR) of 2.5 in the UK group and 2.1 in the Spanish group at the 5% significance level.

Results

There was no significant difference in allele frequencies between UK RA patients and controls. The OR for the presence of the G phenotype was 0.68 (95% CI 0.40–1.16; $P = 0.23$). The OR for the GG genotype was 1.0 (95% CI 0.54–1.85; $P = 1.0$) (Table 1).

A panel of Spanish RA patients was also investigated to look for an association with the CTLA-4 polymorphism. The frequency of the G allele did not differ between patients (G allele present in 52.2%) and controls (G allele present in 56.9%). The OR for the G phenotype was 0.83 (95% CI = 0.51–1.36; $P = 0.42$).

RA is a heterogeneous disease and, therefore, associations may be found in subgroups of patients. No association of the G allele with subgroups stratified for sex, seropositivity and the presence of nodules was demonstrated. A previous study in a German population found that RA patients who were HLA-DRB1*04 positive, particularly those carrying the HLA-DRB1*0401 subtype, were significantly more likely to be homozygous for the GG genotype [9]. HLA data were available for 113 UK RA patients, 77 UK controls, 120 Spanish RA patients and 132 Spanish controls. In the UK individuals for whom HLA data were available, the GG genotype did not occur more frequently in HLA-DRB1*04-positive RA patients ($\chi^2 = 1.66$, $P = 0.44$) or in those carrying the HLA-DRB1*0401 subtype ($\chi^2 = 0.27$, $P = 0.87$) than in controls (Table 2). No association with the GG genotype was demonstrated in the Spanish cohort stratified for HLA.

Discussion

It is proposed that RA is a T-cell-mediated autoimmune disease and autoimmune diseases often co-exist [12]. In murine models of autoimmune disease, genes predisposing to different autoimmune diseases co-localize [13]. It has been postulated, therefore, that certain genes exist

TABLE 1. Frequencies of the cytotoxic T-lymphocyte-associated-4 (CTLA-4) position 49 polymorphism in UK rheumatoid arthritis (RA) patients, UK controls, Spanish RA patients and Spanish controls

Allele/phenotype/genotype	UK patients ($n = 192$)	UK controls ($n = 96$)	Spanish patients ($n = 136$)	Spanish controls ($n = 144$)
Allele frequencies[a]				
G	162 (42%)	89 (46.4%)	85 (31.3%)	94 (32.6%)
A	222 (58%)	103 (53.6%)	187 (68.7%)	194 (67.4%)
Phenotype frequencies[b]				
G positive	124 (65%)	70 (72.9%)	71 (52.2%)	82 (56.9%)
G negative	68 (35%)	26 (27.1%)	65 (47.8%)	62 (43.1%)
Genotype frequencies[c]				
GG	38 (20%)	19 (19.8%)	14 (10.3%)	12 (8.3%)
GA	86 (45%)	51 (53.1%)	57 (41.9%)	70 (48.6%)
AA	68 (35%)	26 (27.1%)	65 (47.8%)	62 (43.1%)

[a]UK subjects: $\chi^2 = 0.90$, $P = 0.34$; Spanish subjects: $\chi^2 = 0.73$, $P = 0.12$.
[b]Odds ratio for G phenotype for UK subjects = 0.68 (95% CI = 0.40–1.16; $P = 0.15$). Odds ratio for G phenotype for Spanish subjects = 0.83 (95% CI = 0.51–1.36; $P = 0.42$).
[c]UK subjects: $\chi^2 = 2.30$, $P = 0.32$; odds ratio for GG phenotype = 1.0 (95% CI = 0.54–1.85; $P = 1.0$). Spanish subjects: $\chi^2 = 1.33$, $P = 0.514$; odds ratio for GG phenotype = 1.26 (95% CI = 0.56–2.84; $P = 0.68$).

which predispose to autoimmunity in general whilst other genes determine the susceptibility of a target organ to an immune response. *CTLA-4* is a prime candidate autoimmunity gene as mutations within the gene leading to alteration in function could have profound effects on the immune system. Defective CTLA-4 expression could result in failure to terminate T-cell activation, leading to an inappropriate and prolonged T-cell response.

The aim of this study was to determine whether a polymorphism in the *CTLA-4* gene is associated with RA. The results show no significant difference in the frequency of the G allele in UK or Spanish patients with RA.

One other study has investigated the association of the CTLA-4 dimorphism with RA in a German population. In the data set as a whole, no significant differences in the distribution of genotypes were seen in RA patients compared with controls. However, further analysis showed that RA patients carrying the HLA-DRB1*0401 subtype were significantly more likely to be homozygous for the GG genotype [9]. We have not replicated this result in either the Spanish or UK groups.

Nistico *et al.* [5] demonstrated linkage and association of the *CTLA-4* gene with IDDM in Spanish and Italian patients, but failed to show evidence of linkage in UK, US or Sardinian patients with IDDM. The lack of association in the UK RA group (OR for G phenotype = 0.68; 95% CI = 0.40–1.16; $P = 0.23$) could be due to an ethnic specificity of the polymorphism such that, in this population, the G allele may not be in as strong linkage disequilibrium with the predisposing allele of the aetiological mutation. Spanish patients with RA were, therefore, compared with controls for the presence of the G allele. Again, however, the frequency of G allele-positive individuals was equal in both groups (OR for G phenotype = 0.83; 95% CI = 0.51–1.36; $P = 0.42$). It should be noted that the Spanish RA patients studied came from north-west Spain and had similar HLA associations as UK RA patients. This is in contrast to other Spanish RA populations where patients are more likely to carry HLA DR1, DR10 or *0405 subtypes [14].

TABLE 2. Frequencies of the cytotoxic T-lymphocyte-associated-4 (CTLA-4) position 49 polymorphism in UK rheumatoid arthritis (RA) patients and UK controls analysed according to HLA-DRB1*04 alleles

	RA			Controls		
HLA	GG *n* (%)	AG *n* (%)	AA *n* (%)	GG *n* (%)	AG *n* (%)	AA *n* (%)
DRB1*04^{+}[a]	12 (11)	41 (36)	28 (25)	6 (8)	14 (18)	6 (8)
DRB1*04^{-}	6 (5)	14 (12)	12 (11)	9 (12)	29 (38)	13 (17)
04,04	7 (6)	15 (13)	6 (5)	–	1 (1)	–
04,X[b]	5 (4)	31 (12)	27 (24)	6 (8)	13 (17)	6 (8)
0401[c]	15 (13)	37 (33)	28 (25)	3 (4)	9 (12)	5 (6)
0402	–	–	–	1 (1)	–	–
0403	–	1 (1)	–	1 (1)	1 (1)	–
0404	3 (3)	8 (7)	3 (3)	–	2 (3)	1 (1)
0405	1 (1)	1 (1)	–	–	–	–
0406	–	2 (2)	–	–	–	–
0407	–	1 (1)	–	1 (1)	3 (4)	–
0408	–	4 (4)	3 (3)	–	–	–

[a]$\chi^2 = 1.66$, $P = 0.44$.
[b]$\chi^2 = 5.42$, $P = 0.07$.
[c]$\chi^2 = 0.27$, $P = 0.87$ (Fisher's exact test).

We have ruled out a significant role for CTLA-4 as the study had the power to detect a gene with a RR of 2.5 and 2.1 (80% power, 5% significance level) in UK and Spanish RA groups, respectively. It is possible that the RR of CTLA-4 in RA is small and that an association has, therefore, been missed.

The results presented herein have not demonstrated an association between a polymorphism of CTLA-4 and RA.

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