Research article

**CTLA-4 +49A/G and CT60 gene polymorphisms in primary Sjögren syndrome**

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**Abstract**

**CTLA-4** encodes cytotoxic T lymphocyte-associated antigen-4, a cell-surface molecule providing a negative signal for T-cell activation. **CTLA-4** gene polymorphisms have been widely studied in connection with genetic susceptibility to various autoimmune diseases, but studies have led to contradictory results in different populations. This case-control study sought to investigate whether **CTLA-4** CT60 and/or +49A/G polymorphisms were involved in the genetic predisposition to primary Sjögren syndrome (pSS). We analysed **CTLA-4** CT60 and +49A/G polymorphisms in a first cohort of 142 patients with pSS (cohort 1) and 241 controls, all of Caucasian origin. A replication study was performed on a second cohort of 139 patients with pSS (cohort 2). In cohort 1, the **CTLA-4** +49A/G*A allele was found on 73% of chromosomes in patients with pSS, compared with 66% in controls (\(p = 0.036; \text{OR} 1.41, 95\% \text{CI} 1.02 \text{to} 1.95\)). No difference in **CTLA-4** CT60 allelic or genotypic distribution was observed between patients (\(n = 142\)) and controls (\(n = 241\)). In the replication cohort, the **CTLA-4** +49A/G*A allele was found on 62% of chromosomes in patients with pSS, compared with 66% in controls (\(p = 0.30; \text{OR} 0.85, 95\% \text{CI} 0.63 \text{to} 1.16\)). Thus, the **CTLA-4** +49A/G*A allele excess among patients from cohort 1 was counterbalanced by its under-representation in cohort 2. When the results from the patients in both cohorts were pooled (\(n = 281\)), there was no difference in **CTLA-4** +49A/G allelic or genotypic distribution in comparison with controls. Our results demonstrate a lack of association between **CTLA-4** CT60 or +49A/G polymorphisms and pSS. Premature conclusions might have been made if a replication study had not been performed. These results illustrate the importance of case-control studies performed on a large number of patients. In fact, sampling bias may account for some contradictory results previously reported for **CTLA-4** association studies in autoimmune diseases.

**Introduction**

Polymorphisms in **CTLA-4**, the gene encoding cytotoxic T lymphocyte-associated antigen-4, have been widely studied in connection with genetic susceptibility to various autoimmune diseases [1], but studies have led to contradictory results in different populations. Among **CTLA-4** gene polymorphisms, a G to A transition at position 49 (+49A/G) of exon 1 leads to an alanine to threonine amino acid substitution at codon 17 in the leader peptide (A17T), and a C to T transition at position 60 (CT60) is located within the 3'-untranslated region [2]. The G allele of +49A/G has been associated with a predisposition to many autoimmune diseases. In contrast, a recent meta-analysis [3] has failed to find a consistent association between **CTLA-4** gene polymorphisms and autoimmune diseases, and the importance of genetic susceptibility to autoimmune diseases remains unclear.

CI = confidence interval; **CTLA-4** = cytotoxic T lymphocyte-associated antigen-4; OR = odds ratio; pSS = primary Sjögren syndrome; SNP = single nucleotide polymorphism.
autoimmune diseases (reviewed in [1]). Both polymorphisms are in linkage disequilibrium, which warrants haplotype analysis in studies of CTLA-4 polymorphisms. The CT60 G allele has been reported to increase susceptibility to several autoimmune diseases [2], and a functional approach provided evidence for lower mRNA levels associated with the CT60 G allele [2].

Downie-Doyle and colleagues have recently reported a significant association of the CTLA-4 +49A/G*A allele and of the CTLA-4 +49A/G*A allele carrier haplotypes with primary Sjögren syndrome (pSS), especially in patients with anti-SSA or anti-SSB antibodies, in a study including 111 Australian patients with pSS and 156 controls [3].

The aim of our study was to investigate in a large case-control study whether CTLA-4 CT60 and/or +49A/G SNPs were involved in genetic predisposition to pSS in French patients.

Materials and methods

Patients

A first cohort of 142 unrelated patients with pSS diagnosed in accordance with the European American consensus group criteria [4] (37% without autoantibodies, 30% with anti-SSA antibodies only, and 33% with both anti-SSA and anti-SSB antibodies) and 241 healthy blood donors were genotyped for the CTLA-4 CT60 and +49A/G polymorphisms in a replication study. The geographical origin and the clinico-biological characteristics of the patients in this second cohort were the same as those in the first. In this second cohort, 27% of patients were anti-SSA and anti-SSB negative, 35% had both anti-SSA only and 38% had both anti-SSA and anti-SSB. All patients and controls were Caucasians and provided informed consent.

Genotyping

After the isolation of genomic DNA from peripheral blood mononuclear cells, CTLA-4 CT60 and +49A/G polymorphisms were genotyped by restriction fragment length polymorphism with the use of BbvI (+49A/G) and NlaIII (CT60).

Statistical analysis

Allelic and genotypic frequencies of CTLA-4 CT60 and +49A/G polymorphisms were compared between patients and controls by using a two-sided χ² test. All genotyped SNPs were in Hardy–Weinberg equilibrium. CTLA-4 (+49A/G or CT60) haplotypes, constructed with the PHASE program, were also examined for association with pSS. P < 0.05 was considered significant.

Results

In the first cohort of patients with pSS, the A allele of the CTLA-4 +49A/G polymorphism was found on 73% of chromosomes in patients with pSS, in comparison with 66% in controls (p = 0.036, odds ratio (OR) 1.41, 95% confidence interval (CI) 1.02 to 1.95; Table 1). No significant difference in CTLA-4 +49A/G*A allele frequencies was observed among subgroups of patients according to their anti-SSB and/or anti-SSA status (Table 1). No difference in CTLA-4 CT60 allelic or genotypic distribution was observed between patients (n = 142) and controls (n = 241). CTLA-4 (+49A/G or CT60) haplotype distribution mirrored the CTLA-4 +49A/G*A allele excess among patients with pSS (A/A 48%, A/G 26%, G/G 26%, G/A 0.4%; in comparison with A/A 45%, A/G 21%, G/G 34% among controls), leading to an excess of +49A/G*A allele carrier haplotypes among patients (p = 0.03, OR 1.41, 95% CI 1.02 to 1.95).

To avoid the possibility of a false positive association of CTLA-4 +49A/G*A with pSS as a result of the somewhat small sam-

| Allelic frequencies of CTLA-4 49A/G polymorphism among patient controls |
|-----------------------------|
| CTLA-4 +49A/G A allele frequencies | pSS | SSA+ and SSB+ | SSA+ only | Ac+ | Ac0 | Controls (n = 241) |
|-----------------------------------|-----|---------------|-----------|-----|-----|-------------------|
| Cohort 1                          |     |               |           |     |     |                   |
| A (Thr)                           | 208 (73) | 68 (72) | 61 (71) | 129 (72) | 79 (76) | 318 (66) | 0.036 | 1.41 (1.02–1.95) |
| G (Ala)                           | 76 (27)  | 26 (28) | 25 (29) | 51 (28) | 25 (24) | 164 (34) | 0.036 | 0.70 (0.51–0.98) |
| Cohort 2                          |     |               |           |     |     |                   |
| A (Thr)                           | 173 (62) | 59 (57) | 66 (67) | 125 (62) | 48 (63) | 318 (66) | NS | 0.85 (0.62–1.15) |
| G (Ala)                           | 105 (38) | 45 (43) | 32 (33) | 77 (38) | 28 (37) | 164 (34) | NS | 1.17 (0.86–1.60) |
| Total                             |     |               |           |     |     |                   |
| A (Thr)                           | 381 (68) | 127 (64) | 127 (69) | 254 (66) | 127 (71) | 318 (66) | NS | 1.08 (0.84–1.40) |
| G (Ala)                           | 181 (32) | 71 (36) | 57 (31) | 128 (34) | 53 (29) | 164 (34) | NS | 0.92 (0.71–1.19) |

Numbers in parentheses are percentages. pSS, primary Sjögren syndrome; Ac+, presence of anti SSB and/or anti-SSA; Ac0, absence of anti-SSA or anti-SSB antibody; CI, confidence interval; NS, not significant.
ple size of our first cohort, and because the CTLA-4 +49A/G*A allele has been only marginally associated with autoimmune diseases compared with the CTLA-4 +49A/G*G allele [1], we performed a replication study on a second independent cohort of 139 patients with pSS. In this second cohort, the CTLA-4 +49A/G*A allele was found on 62% of chromosomes in patients with pSS, compared with 66% in controls (p = 0.30; OR 0.85, 95% CI 0.63 to 1.16; Table 1). Thus, the CTLA-4 +49A/G*A allele excess among patients with pSS from the first cohort was counterbalanced by its under-representation in the second cohort. When the results from the patients in both cohorts were pooled (n = 281), there was no difference in CTLA-4 +49A/G polymorphism allelic or genotypic distribution in comparison with controls (p = 0.53, OR 1.09, 95% CI 0.84 to 1.4; Table 1). The sex ratios among patients (0.97) and controls (0.06) were different. We therefore investigated CTLA-4 +49A/G polymorphism genotypic distribution among males and females in the control group and found that it was not statistically different (p = 0.1), thus excluding any possible gender effect.

Our results therefore demonstrate a lack of association between CTLA-4 CT60 or +49A/G polymorphisms and pSS among Caucasians.

Discussion

The results from our first cohort were very close to those from the study of Downie-Doyle and colleagues [3], with a significant association of pSS with the +49A/G*A allele and with the +49A/G*A allele carriers haplotypes. The association observed in the first cohort, of two haplotypes bearing the same allele (CTLA-4 +49A/G*A), was actually more probably due to the statistical weight of the CTLA-4 +49A/G*A allele than to a true functional effect of two different haplotypes, bearing either CTLA-4 CT60*C or CTLA-4 CT60*T alleles, each having opposite functional effects on CTLA-4 mRNA expression [2].

In fact, our results suggest a false positive association of CTLA-4 +49A/G*A allele with pSS in the first cohort of patients. When data were pooled (cohorts 1 and 2), no significant association was found with the CTLA-4 +49A/G polymorphism in our Caucasian population of patients with pSS. This was not the consequence of different origin or different clinico-biological characteristics of the patients from the two cohorts and could only be the result of a sampling bias. Indeed, the findings observed in our first cohort of patients, as those from Downie-Doyle and colleagues [3], were unexpected because there are only rare examples of association of the CTLA-4A/G*A allele with autoimmune diseases [5-7]. Consequently, we might have made premature conclusions if a replication study had not been performed.

Conclusion

Our study illustrates the necessity to include a large number of patients in genetic case-control studies. In fact, sampling bias may partly account for some contradictory results previously reported for CTLA-4 association studies in autoimmune diseases.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

JEG contributed to the study design, performed the statistical analysis and drafted the manuscript. PL and DC supervised genotyping and contributed to DNA samples collection. MA and CC performed genotyping. NC performed PHASE genotyping and contributed to DNA samples collection. XM and CMR supervised the study design and gave valuable advice to JEG and PL. All authors read and approved the final manuscript.

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