# *Brief Genetics Reports*

# No Major Role for the *CTLA-4* Gene in the Association of Autoimmune Thyroid Disease With IDDM

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IDDM is a T-cell–mediated autoimmune disease depending on both genetic and environmental susceptibility factors. The HLA class II region (IDDM1) and the insulin promoter region (IDDM2) account for ~50 and 10%, respectively, of IDDM genetic risk. At least 15 other IDDM susceptibility markers have been identified by genomewide scanning studies (1). One must consider that IDDM is a heterogeneous disorder that can vary in terms of age at clinical onset, duration of hyperglycemia before strict insulin dependency, existence of familial aggregation, occurrence of complications, and presence of extrapancreatic autoimmune diseases, so that different genes might influence the course or the presentation of the disease. The *CTLA-4* gene, which has been mapped to the IDDM12 locus (2q33), is a good candidate gene in IDDM (2). Apart from recognition of major histocompatibility complex (MHC)/peptide complex by the T-cell receptor (TCR), T-cell activation requires a co-stimulatory signal mediated by CD28/B7 interaction. The *CTLA-4* gene encodes a T-cell surface molecule whose binding to the B7 molecule on antigen-presenting cell delivers a negative signal to the T-cell and can mediate its apoptosis (3). Thus, *CTLA-4* expression on T-cells might well influence the course of an ongoing immune process. *CTLA-4*–deficient mice develop a severe lymphoproliferative disease with multiorgan lymphocytic infiltration and tissue destruction, including the pancreas (4,5). In the same respect, the blockade of the CD28/B7 co-stimulatory signal prevents the occurrence of diabetes but not of insulitis in the NOD mouse model, suggesting that it either promotes a shift of the immune response or makes an additional signal unavailable for the final destructive stage (6). Recently, linkage to IDDM of a point mutation in exon 1 of *CTLA-4* (position 49 A/G) leading to a Thr/Ala substitution in the leader peptide has been demonstrated in multiplex families from Spain and Italy, but not in U.K. and U.S. families (2). Case-control studies have confirmed an association of this polymorphism with IDDM in Belgian and German populations (2,7). Interestingly, *CTLA-4* association has also been reported with Graves' disease, another genetically controlled autoimmune disease (7,8). Because IDDM sometimes occurs together with autoimmune thyroid diseases (AITDs), we investigated whether *CTLA-4* gene polymorphism might favor the development of a polyendocrine disorder in IDDM patients.

We thus analyzed the *CTLA-4* position 49 A/G polymorphism in the following four groups for white populations: group 1, 112 adult-onset IDDM patients (42 women, 70 men; mean age at IDDM onset, 24.9 ± 7.7 years); group 2, 73 patients presenting with Graves' disease alone (59 women, 14 men); group 3, 77 patients presenting with both IDDM and AITD (54 women, 23 men; mean age at IDDM onset, 40.07 ± 19.8 years). AITD was diagnosed on the basis of clinical, biological, and immunological criteria and not solely on the presence of thyroid autoantibodies. The associated AITD was Graves' disease in 41 patients, Hashimoto's thyroiditis in 25 patients, and primary myxedema in 11 patients. Group 4 was made up of 100 healthy blood donors.

HLA class II genotyping was performed using sequence-specific oligonucleotide hybridization after polymerase chain reaction (PCR) amplification of the *DRB1*, *DQA1*, and *DQB1* genes.

*CTLA-4* exon 1 position 49 A/G polymorphism was analyzed using PCR allele-specific dot blot hybridization (2). *CTLA-4* frequencies were determined and odds ratios (ORs) were calculated according to Woolf's formula. The *P* value was defined by $\chi^2$ analysis using a 2 × 2 contingency table or Fisher's exact test when appropriate, and corrected ($P_c$) for the number of comparisons. The level of significance was set to 0.05.

Patients presenting with IDDM or Graves' disease alone were first studied (Table 1). The frequency of the *CTLA-4*/G phenotype was higher in IDDM patients relative to control subjects (67 vs. 53%, respectively) and, conversely, that of *CTLA-4*/A was lower (69.6 vs. 84%). *CTLA-4*/G allele frequency was 48.7% in patients compared with 34.5% in control subjects. This difference reflected a significant increased frequency of the G/G genotype in IDDM patients (30.4 vs. 16% in control subjects) and a decreased frequency of the A/A genotype (33 vs. 47%). In patients with Graves' disease, the fre-

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AITD, autoimmune thyroid disease; MHC, major histocompatibility complex; OR, odds ratio; TCR, T-cell receptor.

TABLE 1
*CTLA-4* exon 1 position 49 polymorphism in control subjects and IDDM and Graves' disease patients

| Nucleotide at position 49 (codon at position 17) | Control subjects $n$ = 100 | IDDM $n$ = 112 | OR | Graves' disease $n$ = 73 | OR | IDDM/AITD $n$ = 77 | OR |
|---|---|---|---|---|---|---|---|
| Phenotype frequencies | | | | | | | |
| G (Ala) | 53 (53.0) | 75 (67.0)* | 1.8 (1.03–3.13) | 50 (68.5)* | 1.9 (1.02–3.62) | 59 (76.6)† | 2.9 (1.5–5.61) |
| A (Thr) | 84 (84.0) | 78 (69.6)‡ | 0.44 (0.22–0.85) | 60 (82.2) | — | 48 (62.4)§ | 0.31 (0.15–0.64) |
| Genotype frequencies | | | | | | | |
| GG (Ala/Ala) | 16 (16.0) | 34 (30.4)‡ | 2.3 (1.17–4.47) | 13 (17.8) | — | 29 (37.6)§ | 3.2 (1.53–6.43) |
| AG (Thr/Ala) | 37 (37.0) | 41 (36.6) | | 37 (50.7) | — | 30 (39.0) | |
| AA (Thr/Thr) | 47 (47.0) | 37 (33.0)* | 0.55 (0.32–0.97) | 23 (31.5) | — | 18 (23.4)† | 0.34 (0.18–0.66) |
| Allele frequencies | | | | | | | |
| G (Ala) | 69 (34.5) | 109 (48.7)‖ | 1.8 (1.21–2.66) | 63 (43.2) | — | 88 (57.2)¶ | 2.5 (1.64–3.9) |
| A (Thr) | 131 (65.5) | 115 (51.3)‖ | 0.55 (0.37–0.82) | 83 (56.8) | — | 66 (42.8)¶ | 0.39 (0.25–0.6) |

Data are $n$ or OR (CI). Only significant risks are given. *$P$ = 0.05, $P_c$ is not significant, †$P_c$ = 0.0045, ‡$P_c$ = 0.05, §$P_c$ = 0.003, ‖$P_c$ = 0.015, ¶$P_c$ < 0.0001.

quency of the *CTLA-4*/G phenotype was also increased as compared with control subjects (68.5 vs. 53%). However, the distribution of *CTLA-4* genotypes was not significantly different between Graves' disease patients and control subjects. These data are in agreement with data reported in Belgian and German populations (2,7), even if some differences were no longer significant after correcting for the number of comparisons made.

In patients with both diseases, the differences were more striking than in those with IDDM or Graves' disease alone (Table 1). This might suggest a stronger contribution of the *CTLA-4* gene in polyendocrine patients than in patients with IDDM or Graves' disease alone. However, the *CTLA-4* frequencies were not significantly different between the three patient groups.

Stratification of patients according to the type of AITD did not evidence major differences between Graves' disease, Hashimoto's thyroiditis, or myxedema groups, except for a strong predisposing effect of the *CTLA-4*/G phenotype in myxedema patients (90.9%, OR = 8.8, CI 1.1–71.9, $P$ = 0.01).

To investigate a possible interaction between *CTLA-4* and *HLA* genes, stratification of patients was done according to their *DR,DQ* phenotype. Because both IDDM and thyroid autoimmune diseases are known to be associated with the DR3 haplotype, we first compared DRB1*03-DQB1*02-DQA1*0501 (positive or negative) patients and matched control subjects. Whereas *CTLA-4* frequencies were similar in DR3$^+$ IDDM, Graves' disease, IDDM/AITD, and control subjects, the *CTLA-4*/G phenotype frequency significantly differed between DR3$^-$ control subjects (50.6%), DR3$^-$ IDDM (74.5%, $P_c$ = 0.03), Graves' disease (72.9%, $P_c$ = 0.045), and IDDM/AITD patients (73.3%, $P_c$ = 0.045). The situation was the opposite when patients and control subjects were matched for the presence of the IDDM-predisposing DRB1*04-DQB1*0302-DQA1*0301 haplotype. The *CTLA-4*/G phenotype was significantly higher in DR4$^+$ IDDM patients with and without AITD than in matched control subjects (67.7% in IDDM, 75% in IDDM/AITD, and 29.4% in control subjects, OR = 5.4, $P_c$ = 0.001, and OR = 7.2, $P_c$ = 0.005, respectively), whereas it did not significantly differ between DR4$^-$ IDDM patients and control subjects. The number of DR4$^+$ Graves' disease patients was too small to allow comparison. Finally, there was no significant difference between non-DR3/non-DR4 patients with IDDM or IDDM/AITD and control subjects (*CTLA-4*/G phenotype frequency = 64.3, 78.9, and 54.7%, respectively, NS).

Because IDDM patients with or without AITD significantly varied with regard to the sex ratio (F/M; 2.34 and 0.6, respectively) and the age at IDDM onset (mean age at onset 40.1 ± 19.8 and 24.9 ± 7.7 years, respectively), comparison of *CTLA-4* phenotypes was made according to these two criteria. No significant differences were observed.

Whether particular genetic factors favor the occurrence of extrapancreatic autoimmunity in IDDM is not well established. Subtle differences in *DR,DQ* haplotypes have been evidenced between IDDM patients with or without associated endocrine autoimmunity, but they cannot fully explain this clinical heterogeneity (9,10). Although the risk conferred by the *CTLA-4*/G phenotype tended to be higher in patients with IDDM plus AITD than in those with IDDM or Graves' disease alone, the difference was not statistically significant. This indicates that the role of *CTLA-4* in the occurrence of polyendocrine autoimmunity is weak, if anything.

Quite surprisingly, when subjects were stratified according to the HLA class II phenotype, the *CTLA-4*/G predisposing effect was maintained in DR3$^-$ but not in DR3$^+$ patients, and maintained in DR4$^+$ but not in DR4$^-$ patients. Apart from IDDM and Graves' disease, the DR3 haplotype is found associated with several organ-specific autoimmune diseases, which might suggest that this haplotype promotes nonspecific immune dysregulation. One could speculate that T-cell activation is less sensitive to the *CTLA-4* regulatory pathway after the engagement of the TCR through DR3/peptide complexes, so that the *CTLA-4* effect is visible in DR3$^-$ but not in DR3$^+$ patients. By contrast, T-cell activation through recognition of DR4/peptide complexes would be more dependent on additional co-stimulatory/regulatory signals to reach the threshold activation level, explaining the clear additional predisposing effect of *CTLA-4* in DR4$^+$ individuals.

Whether *CTLA-4* polymorphism contributes to IDDM and Graves' disease susceptibility by itself or is a marker for another locus in linkage disequilibrium remains unclear. The exon 1 position 49 dimorphism is unlikely to affect the function of the peptide leader. A polymorphic microsatellite (AT)n repeat in the 3′ untranslated region of the *CTLA-4*

gene has been shown to be in strong linkage disequilibrium with the exon 1 dimorphism and might affect mRNA stability. Alternatively, other gene(s) in the vicinity of *CTLA-4* gene, in particular, the homologous and very close *CD28* gene, might be involved in predisposition to autoimmune disorders.

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