

## Association of Single Nucleotide Polymorphisms in Cytotoxic T-Lymphocyte Antigen 4 and Susceptibility to Autoimmune Type 1 Diabetes in Tunisians<sup>∇</sup>

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**In addition to HLA and insulin genes, the costimulatory molecule *CTLA-4* gene is a confirmed type 1 diabetes (T1D) susceptibility gene. Previous studies investigated the association of *CTLA-4* genetic variants with the risk of T1D, but with inconclusive findings. Here, we tested the contributions of common *CTLA-4* gene variants to T1D susceptibility in Tunisian patients and control subjects. The study subjects comprised 228 T1D patients (47.8% females) and 193 unrelated healthy controls (45.6% females). Genotyping for *CTLA-4* CT60A/G (rs3087243), +49A/G (rs231775), and –318C/T (rs5742909) was performed by PCR-restriction fragment length polymorphism (RFLP) analysis. The minor-allele frequencies (MAF) for the three *CTLA-4* variants were significantly higher in T1D patients, and significantly higher frequencies of homozygous +49G/G and homozygous CT60G/G genotypes were seen in patients, which was confirmed by univariate regression analysis (taking the homozygous wild type as a reference). Of the eight possible three-locus *CTLA-4* haplotypes (+49A/G, –318C/T, and CT60A/G) identified, multivariate regression analysis confirmed the positive association of ACG (odds ratio [OR], 1.93; 95% confidence interval [CI], 1.26 to 2.94), GCG (OR, 2.40; 95% CI, 1.11 to 5.21), and GTA (OR, 4.67; 95% CI, 1.52 to 14.39) haplotypes with T1D, after confounding variables were adjusted for. Our results indicate that *CTLA-4* gene variants are associated with increased T1D susceptibility in Tunisian patients, further supporting a central role for altered T-cell costimulation in T1D pathogenesis.**

Type 1 (insulin-dependent) diabetes (T1D) is the most prevalent form of diabetes in children and young adults and results from autoimmune CD4<sup>+</sup> and CD8<sup>+</sup> T-cell-directed destruction of insulin-producing pancreatic  $\beta$  islet cells in genetically susceptible individuals (3, 12), leading to irreversible hyperglycemia and related complications (13). There is a strong genetic component to T1D pathogenesis, evidenced by its clustering in families and by the contributions of a number of susceptibility gene variants to its pathogenesis (10, 12, 29). They include the human leukocyte antigen (HLA) locus, in particular the class II region (DR and DQ), which accounts for 40 to 50% of T1D familial clustering (1, 12, 18), and non-HLA susceptibility loci, several of which were mapped by genome-scanning (11, 29) and/or candidate gene (7, 18, 31) approaches. They include insulin promoter gene variants, which reportedly may modulate immunological tolerance by controlling the expansion of the autoreactive cell pool (26), and the T-cell costimulator cytotoxic T-lymphocyte antigen 4 (*CTLA-4*) transmembrane glycoprotein, which plays a key role in the fine tuning of T-cell immunity (9, 32, 33).

*CTLA-4* is a 40-kDa transmembrane glycoprotein expressed on resting and activated T cells and nonlymphoid cells (33),

and along with the related CD28 costimulatory molecule, it regulates T-cell activation (and is itself primarily mediated by engagement of the T-cell receptor [TCR]) but does recognize major histocompatibility complex (MHC)-bound antigenic peptides (9, 33). *CTLA-4* negatively regulates T-cell activation and effector function, in part by inhibiting Th1 (interleukin 2 [IL-2] and gamma interferon [IFN- $\gamma$ ]) cytokine production and IL-2 receptor  $\alpha$ -chain (p55; Tac) expression by engaging antigen-presenting cell (APC)-bound B7.1 (CD80) and B7.2 (CD86) ligands (9, 33). Functionally, *CTLA-4* attenuates T-cell signaling by interference with intracellular signal transduction events, including TCR signaling, and reduced *CTLA-4* expression and/or activity results in uncontrolled T-cell-associated autoimmunity and lymphoproliferative disease (9, 21). In this regard, it was shown that *CTLA-4* polymorphisms significantly influence the risk of autoimmune diseases, including Graves' disease, systemic lupus erythematosus, autoimmune hypothyroidism, celiac disease, and type 1 diabetes (15, 21, 32).

First observed in Italian subjects (25), and confirmed subsequently by case control and family studies, *CTLA-4* polymorphic variants were linked with T1D pathogenesis (14, 20, 31, 32). While this association was detected in different ethnic groups (14, 23, 30), it appears more likely to be Caucasian selective (10, 29, 33) and absent from non-Caucasians (5, 6, 8, 19, 22). A recent report from the Type I Diabetes Genetics Consortium bearing on 2,300 affected sib pair families demonstrated that among the 24 single nucleotide polymorphisms (SNPs) genotyped in the *CTLA-4* region, only the +49A/G

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TABLE 1. Clinical characteristics of study subjects

Characteristic	Value		<i>P</i> <sup>a</sup>
	Patient ( <i>n</i> = 228)	Controls ( <i>n</i> = 193)	
Gender (M/F) <sup>b</sup>	119/109 (52.2/47.8)	105/88 (54.4/45.6)	0.695
Age at study (yr)	23.7 ± 11.3	24.3 ± 7.1	0.555
T1D duration (yr)	10.5 ± 7.5	NA <sup>c</sup>	NA
Age at T1D onset (yr)	15.7 ± 7.7	NA	NA
Mean BMI (kg/m <sup>2</sup> )	22.2 ± 3.8	24.6 ± 3.4	<0.001
Glucose <sup>d</sup>	13.6 ± 4.7	4.9 ± 1.0	<0.001
Urea <sup>d</sup>	4.9 ± 1.5	4.4 ± 1.1	0.083
Creatinine <sup>3</sup> (μmol/liter)	82.1 ± 18.3	74.6 ± 13.1	0.416
Total cholesterol <sup>d</sup>	4.1 ± 1.3	3.7 ± 0.8	0.020
Triglycerides <sup>d</sup>	1.0 ± 0.6	1.4 ± 0.7	0.022

<sup>a</sup> Student's *t* test (continuous variables) and Pearson's  $\chi^2$  test (categorical variables).

<sup>b</sup> Number (percentage of total) in each group.

<sup>c</sup> NA, not applicable.

<sup>d</sup> Concentration in mmol/liter; the values are means ± standard deviations (SD).

and CT60 SNPs were replicated in the nine combined collections (27). In the present study, we investigated the association of three common *CTLA-4* SNPs (−318C/T; +49A/G, and CT60A/G) and the corresponding haplotypes with T1D in Tunisian Arab patients.

#### MATERIALS AND METHODS

**Subjects.** The study subjects comprised 228 unrelated T1D patients (47.8% females; mean age, 16.4 ± 7.7 years) and 193 university students and healthy children, who served as controls (45.6% females; mean age, 28.2 ± 5.8 years) (Table 1). Patients with other forms of diabetes were excluded. The diagnosis of T1D was made based on both clinical features and laboratory data. The inclusion criteria for the recruitment of T1D patients were the presence of diabetic ketosis at onset, dependence on insulin therapy for controlling hyperglycemia, and testing positive for at least one of the anti-islet autoantibodies. T1D patients were not obese (body mass index [BMI], 22.2 ± 3.8 kg/m<sup>2</sup>), were free of concomitant complications, and did not receive additional medication.

Control subjects had normal glucose tolerance and were matched for gender (*P* = 0.695) and age (*P* = 0.555), and none reported a family history of T1D or other autoimmune diseases. While significantly higher cholesterol (*P* = 0.020) and lower BMI (*P* < 0.001) and triglycerides (*P* = 0.022) were recorded for T1D patients (Table 1), they were within the reference ranges. All patients and controls were Tunisian Arabs and originated in central Tunisia: they were asked to sign a consent form according to the study protocol, and all institutional ethics requirements were met.

***CTLA-4* genotyping.** Three SNPs in the *CTLA-4* gene, CT60A/G (rs3087243), +49A/G (rs231775), and −318C/T (rs5742909), were genotyped in all samples, as previously described (15). In order to validate the PCR-restriction fragment length polymorphism (RFLP) results, 75 patient and 75 control specimens were independently genotyped by direct DNA sequencing. Complete concordance was seen in both genotyping methods, and no single discrepancy was observed.

**Data analysis.** The allelic frequencies were determined by the gene-counting method, using HLAStat 2000 software (courtesy of M. Busson, Hôpital St. Louis, Paris, France). This also computed the *P* values (Fisher's exact probability test), and odds ratios (OR). Deviation from Hardy-Weinberg equilibrium (HWE) was analyzed by Pearson's  $\chi^2$  test using HPlus 2.5 software (<http://qge.fhcr.org/hplus>). Three-locus (+49A/G, −318C/T, and CT60A/G) *CTLA-4* haplotype frequency determination was done by the maximum-likelihood method, using HPlus 2.5. To minimize the possibility of spurious association or chance findings, *P* values were corrected for the number of different haplotypes tested (*P*<sub>c</sub>) using the Bonferroni inequality method [*P*<sub>c</sub> = 1 − (1 − *P*)<sup>*n*</sup>, where *n* is the number of comparisons]. These tests are used when several dependent or independent tests are performed simultaneously and the individual *P* value may not be appropriate for all comparisons.

Taking healthy subjects as references, univariate and later multivariate regression analyses were performed to estimate the OR and 95% confidence intervals (CI) using HPlus 2.5 and HAPStat haplotype analysis software (<http://www.bios.unc.edu/~lin/hapstat>). The structure of the model used was checked using the

confidence interval for the parameters tested; a CI of 0.0 meant removal of that parameter from the model. The confounding variables included in the final model were BMI, urea, total cholesterol, and triglycerides. Additional statistical analysis was performed with the SPSS version 17.0 for Windows statistical package (SPSS Inc., Chicago, IL).

#### RESULTS

**Genotype analysis.** The genotype frequency distributions of +49A/G (*P* = 0.102;  $\chi^2$  = 2.682) and CT60A/G (*P* = 0.083;  $\chi^2$  = 2.998), but not −318C/T (*P* = 0.001;  $\chi^2$  = 14.123), were in Hardy-Weinberg equilibrium in the controls. The minor-allele frequencies (MAF) for the −318C/T (*P* = 0.008), +49A/G (*P* = 0.002), and CT60A/G (*P* = 0.002) SNPs were significantly higher in T1D patients (Table 2). Varied distributions of *CTLA-4* genotypes were noted between T1D patients and controls, with significantly higher frequencies of homozygous +49G/G (20.6% versus 10.4%; *P* = 0.006) and homozygous CT60G/G (13.6% versus 6.2%; *P* = 0.020) genotypes seen in T1D patients (Table 2). Taking the homozygous wild type as a reference (OR = 1.00), univariate regression analysis confirmed the association of +49G/G (*P* = 0.028; OR [95% CI] =

TABLE 2. Allele and genotype frequencies of *CTLA-4* polymorphisms

SNP	Allele/genotype	T1D patients <sup>a</sup>	Controls <sup>a</sup>	<i>P</i> <sup>b</sup>
−318C/T	T allele	84 (18.4)	45 (11.7)	0.008
	C/C	159 (69.7)	156 (80.8)	0.012
	C/T	52 (22.8)	29 (15.0)	0.058
	T/T	17 (7.5)	8 (4.1)	0.287
+49A/G	G allele	177 (38.8)	109 (28.2)	0.002
	A/A	98 (43.0)	104 (53.9)	0.033
	A/G	83 (36.4)	69 (35.8)	0.970
	G/G	47 (20.6)	20 (10.4)	0.006
CT60A/G	G allele	137 (30.0)	79 (20.4)	0.002
	A/A	122 (53.5)	126 (65.3)	0.019
	A/G	75 (32.9)	55 (28.5)	0.386
	G/G	31 (13.6)	12 (6.2)	0.020

<sup>a</sup> Number (percent of total).

<sup>b</sup> Pearson's  $\chi^2$  test.

TABLE 3. Univariate odds ratios for association of CTLA4 variants with T1D

SNP	Genotype	<i>P</i>	OR (95% CI)
-318C/T	C/C	0.106	1.00 (reference)
	C/T	0.034	1.75 (1.04–2.93)
	T/T	0.673	1.22 (0.48–3.13)
+49A/G	A/A	0.089	1.00 (reference)
	A/G	0.546	1.14 (0.74–1.76)
	G/G	0.028	2.04 (1.08–3.84)
CT60A/G	A/A	0.096	1.00 (reference)
	A/G	0.366	1.23 (0.79–1.90)
	G/G	0.035	2.26 (1.06–4.81)

2.04 [1.08 to 3.84]) and CT60G/G ( $P = 0.035$ ; OR [95% CI] = 2.26 [1.06 to 4.81]) with increased odds of T1D presence (Table 3).

**Haplotype distribution.** Of the eight three-locus (+49A/G, -318C/T, and CT60A/G) *CTLA-4* haplotypes identified, the frequencies of ACG ( $P = 0.037$ ) and GTA ( $P = 0.003$ ) were higher, while that of ACA ( $P < 0.001$ ) was lower, among T1D patients than among control subjects (Table 4). Following adjustment of  $P$  values (Bonferroni correction), differences were significant for only the GTA haplotype ( $P_c = 0.024$ ), which was higher, and the ACA haplotype ( $P < 0.001$ ), which was lower among T1D patients, thereby conferring T1D-susceptible and -protective natures on these haplotypes, respectively (Table 4).

**Regression analysis.** Univariate and multivariate regression analyses confirmed the positive association of the ACG ( $P = 0.006$ ) and GTA ( $P = 0.010$ ) haplotypes with T1D and, in addition, identified GCA ( $P = 0.048$ ) and GCG ( $P = 0.026$ ) as positively associated with T1D. Multivariate analysis confirmed the positive association of the ACG ( $P = 0.002$ ; OR, 1.93; 95% CI, 1.26 to 2.94), GCG ( $P = 0.027$ ; OR, 2.40; 95% CI, 1.11 to 5.21), and GTA ( $P = 0.007$ ; OR, 4.67; 95% CI, 1.52 to 14.39) haplotypes with T1D, after the confounding variables BMI, urea, total cholesterol, and triglycerides were adjusted for (Table 5).

## DISCUSSION

Optimal T-cell activation requires two signals, one provided by TCR-CD3 complex ligation to antigenic fragments bound to MHC class II molecules on the surfaces of APCs, and a second, non-antigen-specific (costimulatory) signal provided by costimulatory molecules that synergize with TCR-CD3 signals in enhancing T-cell activation. The latter include CTLA-4 and the related costimulatory molecule CD28, which are located on chromosome 2q33 and play key roles in driving sustained T-cell activation (33). Accordingly, defective or inappropriate costimulatory signaling precipitates a state of anergy, in which the cell becomes refractory to further stimulation, and induction of apoptosis (9, 33). While CTLA-4 and CD28 bind the same ligands (CD80 and CD86), CD28 delivers positive while CTLA-4 provides inhibitory costimulatory signals (9, 33). Accordingly, defective CTLA-4 signaling results in uncontrolled T-cell activation and the pathogenesis of lymphoproliferative and autoimmune disease (21, 32), including T1D.

Previously, we reported on the positive (DRB1\*030101 and

TABLE 4. CTLA-4 Haplotype distribution

Haplotype <sup>a</sup>	Haplotype frequency $\pm$ SE		<i>P</i>	<i>P</i> <sub>c</sub> <sup>b</sup>
	T1D patients	Healthy subjects		
ACA	0.366 $\pm$ 0.022	0.548 $\pm$ 0.017	<0.001	<0.001
GCA	0.205 $\pm$ 0.025	0.177 $\pm$ 0.029	0.360	0.972
ACG	0.157 $\pm$ 0.030	0.107 $\pm$ 0.032	0.037	0.260
GCG	0.087 $\pm$ 0.017	0.051 $\pm$ 0.017	0.060	0.390
ATA	0.061 $\pm$ 0.020	0.049 $\pm$ 0.031	0.486	0.995
GTG	0.029 $\pm$ 0.014	0.032 $\pm$ 0.019	0.987	1.000
GTA	0.067 $\pm$ 0.015	0.022 $\pm$ 0.016	0.003	0.024
ATG	0.027 $\pm$ 0.061	0.014 $\pm$ 0.096	0.259	0.909

<sup>a</sup> CTLA4 haplotype (+49A/G, -318C/T, or CT60A/G) frequency determined by the maximum-likelihood method; haplotypes were coded according to the allele at each locus.

<sup>b</sup>  $P_c$ , corrected  $P$ , calculated by the Bonferroni method [ $P_c = 1 - (1 - P)^n$ ], where  $n$  is the number of comparisons.

DQB1\*0302) and negative (DRB1\*070101-DRB1\*110101 and DQB1\*030101-DQB1\*060101) association of HLA class II alleles with the presence of T1D and identified both T1D-susceptible (DRB1\*030101-DQB1\*0201 and DRB1\*040101-DQB1\*0302) and T1D-protective (DRB1\*070101-DQB1\*0201) haplotypes (28). The genetic associations between the *CTLA-4* polymorphic variants -318C/T, +49A/G, and CT60A/G were previously investigated in different ethnic groups, but with inconsistent findings (6, 17, 19, 30, 35). CTLA-4 is now among the five replicated and established non-HLA loci, together with INS, PTPN22, IL2RA, and IFIH1 (27). Our findings confirm the association of all three *CTLA-4* variants with T1D, evidenced by enrichment of the mutant allele in patients and by the identification of specific (three-locus) haplotypes associated with increased T1D risk among Tunisians. Our working hypothesis is that defective (negative) signaling imparted by mutant susceptibility to a *CTLA-4* variant(s) augments T-cell destruction of  $\beta$  islet cells by increasing interaction of the (positive) costimulatory signal CD28 with the shared B7, resulting in augmentation of the activities of TCR-associated protein (tyrosine) kinases. This results in dysregulated T-cell immunity and hence infiltration of autoreactive T cells into the pancreas, leading to  $\beta$  islet cell destruction, as has been suggested (32, 33).

We found that the +49A/G at-risk G allele (OR = 1.61; 95% CI = 1.20 to 2.15), and the homozygous G/G genotype (OR = 2.25; 95% CI = 1.27 to 3.87) were positively associated with increased T1D risk. Previous studies on the +49A/G *CTLA-4* gene variant and T1D yielded inconsistent associations, suggesting an ethnic contribution to the association of the +49A/G variant with T1D pathogenesis. This was exemplified by the enrichment of the +49G at-risk allele and G/G genotype in T1D in Chinese patients (17), but mostly in patients of Caucasian descent, including Lebanese (34), Dutch (35), northern European (14), and U.S. whites (10), but not in non-Caucasians, such as Chileans (5), north Indians (6), and Koreans (19), or in Portuguese (22). These apparent discrepancies may be attributed to several factors, including differences in genetic background (16, 23), possible linkage to HLA-susceptible haplotypes (19), and patient selection, as T1D is often accompanied by other autoimmune conditions (16, 17, 35). In support of this was the finding that the association of a

TABLE 5. CTLA-4 haplotype distribution

Haplotype <sup>a</sup>	Univariate			Multivariate		
	Z score	P	OR (95% CI)	Z score	P	aOR <sup>b</sup> (95% CI)
ACA			1.00 (reference)			1.00 (reference)
GCA	1.98	0.048	1.55 (1.00–2.40)	1.91	0.056	1.52 (0.99–2.34)
ACG	2.73	0.006	1.89 (1.20–2.98)	3.04	0.002	1.93 (1.26–2.94)
GCG	2.23	0.026	2.28 (1.10–4.72)	2.22	0.027	2.40 (1.11–5.21)
ATA	0.88	0.376	1.61 (0.56–4.65)	0.94	0.345	1.61 (0.60–4.30)
GTG	–0.15	0.878	0.90 (0.23–3.52)	–0.19	0.851	0.85 (0.16–4.60)
GTA	2.57	0.010	4.52 (1.43–14.26)	2.69	0.007	4.67 (1.52–14.39)
ATG	0.94	0.345	2.75 (0.34–22.43)	1.00	0.319	2.68 (0.39–18.61)

<sup>a</sup> CTLA-4 haplotype (+49A/G, –318C/T, or CT60A/G) frequency determined by the maximum-likelihood method; haplotypes were coded according to the allele (wild type or mutant) at each locus.

<sup>b</sup> aOR, adjusted odds ratios (adjusted for BMI, urea, total cholesterol, and triglycerides).

+49A/G variant in non-Caucasians was reported in Japanese (16) and Chinese (17) patients with autoimmune thyroiditis.

The CT60A/G variant was also associated with increased T1D risk in Tunisian patients, and an increased frequency of the G at-risk allele (OR = 1.67; 95% CI = 1.21 to 2.29) and G/G genotype carriers (OR = 2.37; 95% CI = 1.17 to 4.60) was seen in the T1D patients compared to nondiabetic controls, in agreement with recently published studies on Caucasians (14, 24, 35). In contrast to the negative result seen in non-Caucasians (6), a positive association was reported for Chinese (17). It should be noted that this was seen in T1D complicated with autoimmunity (autoimmune thyroiditis), thereby calling into question the contribution of the CT60A/G variant to T1D pathogenesis in the absence of other contributing conditions, as was also shown elsewhere (24, 35).

A limited number of studies have investigated the association of the CTLA-4 –318C/T variant with T1D, but with inconclusive findings. Baniasadi et al. showed that the –318T allele conferred T1D susceptibility on north Indians (6), while Balic et al. reported that the –318C/T variant did not influence the overall risk of T1D in Caucasians (5). In this study, we found no significant association of CTLA-4 –318C/T polymorphism with T1D in Tunisian patients, in agreement with published reports on populations with diverse ethnic backgrounds (5, 6, 8, 19). Furthermore, a recent meta-analysis of 5,637 T1D patients and 6,759 controls demonstrated no association of the –318 variant with T1D (OR = 0.92; 95% CI = 0.45 to 1.89) after several confounders were controlled for (20). Whereas the –318C/T variant was previously linked to disorders of altered immunity (2, 4), its contribution to T1D pathogenesis (if any) remains questionable.

The contributions of CTLA-4 variants to T1D pathogenesis were further supported by three-locus (+49A/G, –318C/T, and CT60A/G) haplotype analysis, with the ACG (OR = 1.93; 95% CI = 1.26 to 2.94), GCG (OR = 2.40; 95% CI = 1.11 to 5.21), and GTA (OR = 4.67; 95% CI = 1.52 to 14.39) haplotypes positively associated with T1D. A limited number of studies identified CTLA-4 haplotypes associated with T1D. Like us, Zhernakova et al. identified +49G/CT60G-containing haplotypes as overrepresented in Dutch T1D patients (35). In contrast, Baniasadi et al. demonstrated increased prevalence of the +49A/–318T/CT60G haplotype in north Indian T1D patients (6), which in our hands was present at lower but

comparable frequencies between patients ( $0.027 \pm 0.061$ ) and control subjects ( $0.014 \pm 0.096$ ;  $P_c = 0.909$ ).

In conclusion, in (North African) Tunisians, CTLA-4 +49A/G and CT60A/G, more so than the –318C/T polymorphism, are associated with increased risk of T1D susceptibility. While our data do not rule out a contribution of the –318C/T variant to the risk of T1D development, they underscore the need for larger studies (including meta-analyses) to elucidate the effect of the CTLA-4 region on the development of T1D. The strengths of this study lie in being the first to examine the association of CTLA-4 variants with T1D in the ethnically homogeneous Tunisian Arabs and in identifying T1D-associated CTLA-4 haplotypes. This study has shortcomings, namely, that it was underpowered (64.1% power), owing to the difficulty in collecting sufficient T1D cases due to the low incidence of T1D in Tunisia (6.76 to 6.95/100,000) (4). We also did not correlate CTLA-4 genotypes (and haplotypes) with soluble and membrane-bound CTLA-4 levels, thereby calling into question the functional relevance of the variants analyzed. Furthermore, the potential interaction of the CTLA-4 polymorphisms studied with other nearby or distant functional gene variants, in particular HLA, remains to be seen. Despite these limitations, the association of CTLA-4 polymorphisms with T1D susceptibility strengthens our understanding of the link between dysregulated (T-cell) immunity and T1D pathogenesis.

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We declare that we have no competing interests.

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