RESEARCH ARTICLE

Identification of specific tumor necrosis factor-α-susceptible and -protective haplotypes associated with the risk of type 1 diabetes

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ABSTRACT. *Aim*. We investigated the association of tumor necrosis factor (TNF)α gene polymorphism with type 1 diabetes (T1D). *Methods*. TNF-α -1031T/C, -863C/A, -857C/T, -376G/A, -308G/A, -238G/A, and +488G/A single nucleotide polymorphisms (SNPs) were assessed in 198 T1DM patients and 180 age-and gender-matched, normoglycemic control subjects using PCR-restriction fragment length polymorphism (RFLP). *Results*. Higher frequencies of -863A ($p = 8.0 \times 10^{-6}$), -857T ($p = 1.4 \times 10^{-4}$), and -238A ($p = 0.002$) alleles were seen in T1D patients than in the control group. Significant differences were noted in the distribution of -863T/C, -857C/T, -376G/A, -308G/A, and -238G/A genotypes between patients and controls. Haploview analysis revealed high linkage disequilibrium (LD) between the -376G/A and -308G/A SNPs, but this was lower between the other polymorphisms. Five-locus TNFα haplotypes were constructed based on the prevalence of individual SNPs and the LD between them. An increased frequency of CTGGG, CCGAG, and ACGGG haplotypes, and a reduced frequency of the CCGGG haplotype was seen in patients. When the Bonferroni correction was applied, differences were significant for the CTGGG ($Pc = 1.4 \times 10^{-3}$), CCGAG ($Pc = 0.023$), and ACGGG ($Pc = 1.2 \times 10^{-3}$) haplotypes which were greater, and the CCGGG haplotype ($Pc = 3.8 \times 10^{-5}$) which was smaller, among T1D patients, thereby conferring susceptibility to and protection from T1D, respectively. *Conclusion*. These results demonstrate that TNF-α polymorphisms, in particular -863C/A, -857C/T, and -238G/A, are significantly associated with T1D. Additional studies, on other racial groups, are needed to confirm our findings.

Keywords: tumor necrosis factor, polymorphisms, type 1 diabetes

Type 1 diabetes (T1D) is an autoimmune disease characterized by autoimmune CD4+ and CD8+ T cell-directed destruction of insulin-secreting, pancreatic islet β cells [1], resulting in irreversible hyperglycemia and specific complications [2]. T1D is multi-factorial, and susceptibility to it is determined by environmental, immunological, and genetic factors [1, 3], the latter including HLA class II DRB1 and DQB1 loci [4, 5], and class I A, B, and C loci [6]. Additional genes lying outside the HLA region (class III MHC), which include those for tumor necrosis factor (TNF) α and lymphotoxin α (LTA)/TNF-β, have also been implicated in the pathogenesis of T1D. TNF-α and LTA/TNF-β genes are polymorphic, and variants of TNFα and LTA/TNF-β have been previously linked with diseases associated with altered immunity, such as asthma [7, 8], rheumatoid arthritis [7, 9], inflammatory bowel disease [10, 11], and T1D [12-15].

TNF-α is a major pro-inflammatory cytokine produced by mononuclear phagocytes, natural killer (NK) cells, and antigen-activated T cells [16], and is implicated in islet β-cell destruction, leading to the development of T1D [17, 18]. In this regard, it was shown that IFN-γ and TNF-α, secreted by autoreactive CD4+ T lymphocytes and macrophages, upregulate the expression of pro-apoptotic genes, leading to apoptosis and necrosis of pancreatic islet β cells [18, 19]. TNF-α secretion is genetically-controlled [20, 21], and TNF-α variants associated with altered TNF-α expression, including the promoter -238G/A, -308G/A, -863C/T, and -1031T/C polymorphisms, have been reported [20, 22, 23]. Given their close proximity and reported linkage disequilibrium (LD) with HLA class II genes, TNF-α polymorphisms appear to be linked to T1D [13, 24]. However, the functional significance of this remains controversial, as

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exemplified by the contrasting association of particular TNF-α variants with T1D, after adjustment for LD with HLA class II (DRB1-DQB1) alleles [24-26].

Previous studies investigating the association between TNF-α gene variants and T1D have been reported by some [13-15, 27], but not all studies [26, 28], and an ethnic contribution to the association of TNF-α variants with T1D have been suggested [12, 29]. More recently, we reported on the lack of association of the -308G/A variant with T1D in Bahraini subjects [26]. Here, we investigate the contribution of the TNF-α variants -1031T/C, -863C/A, -857C/T, -376G/A, -308G/A, -238G/A, and +488G/A to the pathogenesis of T1D in 198 T1DM patients and 180 age-and gender-matched control subjects.

DONORS AND METHODS

Patients

Study participants comprised 198 unrelated T1D patients (129 males and 69 females; age 16.4 ± 6.3 years) *(table 1)*. The diagnosis of T1D was in accordance with clinical features and laboratory findings. All patients were ketosis-prone, lacked endogenous insulin production, and were dependent on insulin for controlling hyperglycemia. Patients were not obese (BMI = 19.9 ± 3.9 kg/m^2), were free of concomitant complications, and were not receiving additional treatment at the time of blood collection. Patients with other forms of diabetes (LADA, juvenile type 2 diabetes) were excluded. Control subjects consisted of 180 students and healthy children [129 males and 51 females; age (mean ± SE) 17.3 ± 7.1 years], who had normal glucose tolerance and no family history of T1D, or any other autoimmune diseases *(table 1)*. All patients and control subjects were Tunisian Arabs, originating in Central Tunisia, and were asked to sign a consent form in accordance with the study protocol; all institutional, ethics requirements were met.

Table 1
Clinical characteristics of study subjects

Characteristic	Patient (n = 198)	Controls (n = 180)	P[1]
Gender (M:F)	129:69 (65.2:34.8)[2]	129:51 (71.7:28.3)	0.147
Age at study (years)	16.4 ± 6.3	17.3 ± 7.1	0.555
T1D duration (years)	10.5 ± 7.5	N/A [3]	N/A
Age at T1D onset (years)	10.2 ± 6.8	N/A	N/A
Mean BMI (kg/m^2)	19.9 ± 3.9	25.3 ± 3.1	0.001
Glucose[4]	12.3 ± 5.7	5.2 ± 1.3	< 0.001
HbA1c (%)	9.8 ± 2.4	5.0 ± 1.0	< 0.001
Urea[4]	4.6 ± 1.5	4.5 ± 1.1	0.481
Creatinine[3] (μmol/L)	82.1 ± 18.3	74.6 ± 13.1	.416

[1] Student's *t*-test (continuous variables), Pearson's chi square test (categorical variables).
[2] Number (percent of total) in each group.
[3] NA: not applicable.
[4] Concentration in mmol/L; values indicate mean ± SD.

TNF-α gene polymorphisms

TNF-α genotyping was performed using PCR-restriction fragment-length polymorphism (RFLP) analysis. DNA was amplified using forward and reverse primers (Thermo Scientific, Ulm, Germany) *(table 2)*. Genotype was determined by agarose gel electrophoresis following restriction enzyme digestion *(table 2)*. Quality control measures comprised direct DNA re-sequencing of patient (n = 50) and control (n = 75) specimens (ABI 3130xl Genetic Analyzer). The genotyping call rate exceeded 98%, with no significant differences between cases and control samples.

Statistical analysis

Statistical analysis was performed on SPSS v. 17.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as percentage of total (categorical variables) or mean ± SD (continuous variables). Student's *t*-test was used in testing differences in means, and Pearson χ^2 or Fisher's exact test was used in assessing inter-group significance. Allele frequencies were calculated using the gene-counting method; each polymorphism was tested for Hardy-Weinberg equilibrium using χ^2 goodness-of-fit test using HPlus 2.5 software (http://qge.fhcrc.org/hplus).

Table 2
TNF-α primers

SNP	Primer	Sequence (5' → 3')	Enzyme
-1031T/C	Sense	TAT GTG ATG GAC TCA CCA GGT	B*bs* I
	Anti-sense	CCT CTA CAT GGC CCT GTC TT	
-863C/A	Sense	GGC TCT GAG GAA TGG GTT AC	T*ai* I
	Anti-sense	CTA CAT GGC CCT GTC TTC GTT ACG	
-857C/T	Sense	GGC TCT GAG GAA TGG GTT AC	T*ai* I
	Anti-sense	CCT CTA CAT GGC CCT GTC TAC	
-376 G/A	Sense	CCC CGT TTT CTC TCC CTC AA	T*sp509* I
	Anti-sense	TGT GGT CTG TTT CCT TCT AA	
-308G/A	Sense	GAG GCA ATA GGT TTT GAG GGC CAT	N*co* I
	Anti-sense	GGG ACA CAC AAG CAT CAA G	
-238G/A	Sense	AAA CAG ACC ACA GAC CTG GTC	B*am* HI
	Anti-sense	CTC ACA CTC CCC ATC CTC CCG GAT C	
+488G/A	Sense	GCC AGA CAT CCT GTC TCT CC	N*la* III
	Anti-sense	CAG AGG GAA GAG GTG AGT GC	

All analyses were conducted assuming an additive genetic effect, as it is the most conservative mode. LD analysis and haplotypes reconstruction was performed using Haploview 4.1 (http://www.broad.mit.edu/mpg/haploview). The Boneferroni multiple-comparison correction method was employed in calculating the corrected *P* (*Pc*) value, as per: $Pc = 1 - (1-P)^n$, where n = number of comparisons. Logistic regression analysis was performed in order to determine the odds ratios (OR) and 95% confidence intervals (95% CI) associated with the RM risk, taking the control women as the reference group. Statistical significance was set at $p < 0.05$.

RESULTS

Study population

Table 1 summarizes the characteristics of the study participants. T1D patients and control subjects were matched for gender (p = 0.147) and age (p = 0.555), controls having higher BMI than patients (p = 0.001). As expected, elevated fasting glucose ($p < 0.001$) and HbA1c ($p < 0.001$) was seen in patients. A higher incidence of anti-GAD (87/198 *vs* 0/180; $p < 0.001$), ICA (72/198 *vs* 0/180; $p < 0.001$), and combined anti-GAD/ICA (32/198 *vs* 0/180; $p < 0.001$) was seen in patients than controls, respectively. The mean age at T1D onset was 10.2 ± 6.8 years, with an average T1D duration of 10.5 ± 7.5 years.

Allele and genotype analysis

The distribution of TNF-α -1031T/C (p = 0.73), -863C/A (p = 0.45), -857C/T (p = 0.06), -376G/A (p = 0.31), -308G/A (p = 0.13), -238G/A (p = 0.47), and +488G/A (p = 1.00) genotypes were in Hardy Weinberg equilibrium. Haploview analysis revealed high LD between the -376G/A and -308G/A SNPs, but this was lower between the other polymorphisms *(figure 1)*.

The frequencies of -863A ($p = 8.0 \times 10^{-6}$), -857T ($p = 1.4 \times 10^{-4}$), and -238A (p = 0.002) alleles were higher in T1D patients than in the control group (*table 3*). Significant differences were seen in the distribution of the -863T/C ($p = 1.1 \times 10^{-6}$), -857C/T ($p = 3.1 \times 10^{-7}$), -376G/A (p = 0.017), -308G/A (p = 0.020), and -238G/A ($p = 3.0 \times 10^{-4}$) genotypes between T1D patients and control subjects *(table 4)*.

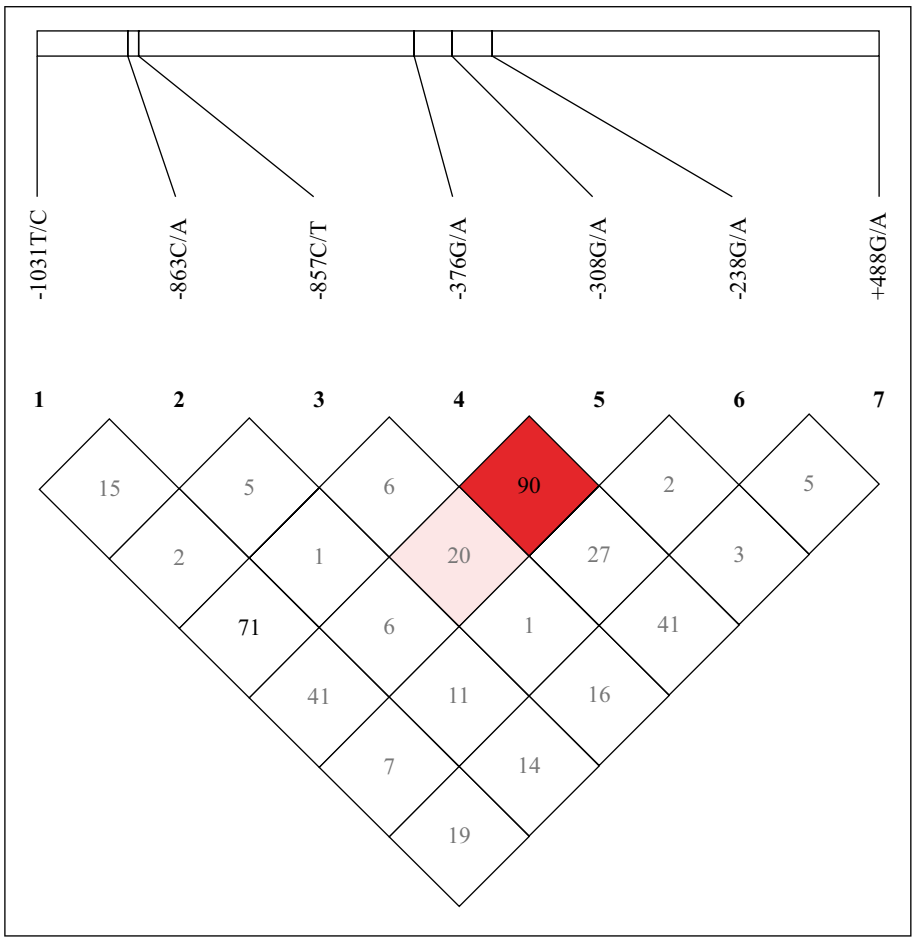

Figure 1

Haploview graph of the seven SNPs in the TNF gene analyzed. Light red/pink block, D' (normalized linkage disequilibrium measure or D) < 1.0, with logarithm of odds (LOD) score > 2.0; blue blocks, D' = 1.0 but LOD < 2.0; white blocks, D' < 1.0 with LOD < 2.0; numbers in blocks denoting the D' value. The genomic organization is depicted above the LD plot. LOD being defined as $\log_{10}(L1/L0)$, where L1 = likelihood of the data under linkage disequilibrium, and H0 = likelihood of the data under linkage equilibrium. D' is calculated as per: D' = (D) divided by the theoretical maximum for the allele frequencies observed.

Table 3
TNF-α SNPs allele frequencies

	Minor allele	Cases[1]	Controls[1]	P value[2]	Pc[4]	OR (95% CI)
-1031 T/C	C	78 (19.7)	71 (19.7)	0.948	1.00	0.995 (0.69-1.42)
-863 C/A	A	54 (13.6)	12 (3.3)	1.0×10^{-6}	8.0×10^{-6}	4.61 (2.38-8.37)
-857 C/T	T	102 (25.8)	38 (10.6)	1.8×10^{-5}	1.4×10^{-4}	2.38 (1.59-3.51)
-376 G/A	A	46 (11.6)	21 (5.8)	7.3×10^{-3}	0.057	2.09 (1.23-3.47)
-308 G/A	A	80 (20.2)	45 (12.5)	0.010	0.077	1.72 (1.15-2.54)
-238 G/A	A	68 (17.2)	28 (7.8)	1.9×10^{-4}	0.002	2.39 (1.51-3.71)
+488 G/A	A	20 (5.1)	12 (3.3)	0.338	0.962	1.52 (0.73-3.08)

[1] Study subjects comprised 198 cases and 180 control subjects.
[2] Fisher's exact test.
[3] Number (percentage of total).
[4] Pc = corrected *P* as per Bonferroni correction.

Table 4
Distribution of TNF-α genotypes

SNP	Genotype	Cases	Controls	P[1]
-1031T/C	T/T	126 (63.6)	114 (63.3)	0.988
	T/C	66 (33.3)	61 (33.9)	
	C/C	6 (3.0)	5 (2.8)	
-863 C/A	C/C	145 (73.2)	168 (93.3)	1.1×10^{-6}
	C/A	52 (26.3)	12 (6.6)	
	A/A	1 (0.5)	0 (0.0)	
-857C/T	C/C	110 (55.6)	142 (78.9)	3.1×10^{-7}
	C/T	74 (37.4)	38 (21.1)	
	T/T	14 (7.1)	0 (0.0)	
-376G/A	G/G	156 (78.7)	159 (88.3)	0.017
	G/A	38 (19.2)	21 (11.7)	
	A/A	4 (2.0)	0 (0.0)	
-308G/A	G/G	123 (62.1)	131 (75.6)	0.020
	G/A	70 (35.4)	41 (23.3)	
	A/A	5 (2.5)	2 (1.1)	
-238G/A	G/G	133 (67.2)	153 (85.0)	3.0×10^{-4}
	G/A	62 (31.3)	26 (14.4)	
	A/A	3 (1.5)	1 (0.6)	
+488G/A	G/G	178 (89.9)	168 (93.3)	0.271
	G/A	20 (10.1)	12 (6.7)	

[1] One-way ANOVA.

Table 5
Distribution of common TNF-α haplotypes

TNF-α haplotype[1]	Cases	Controls	P	(Pc)[2]	aOR (95% CI)[4]
C C G G G	156 (0.394)	250 (0.694)	4.8×10^{-6}	3.8×10^{-5}	0.29 (0.21 – 0.40)
C C G G A	26 (0.066)	31 (0.086)	0.378	0.978	0.748 (0.43-1.30)
C T G G G	58 (0.146)	21 (0.058)	1.7×10^{-4}	1.4×10^{-3}	2.84 (1.63-4.79)
C C A A G	17 (0.043)	16 (0.044)	0.977	1.00	0.944(0.46-1.92)
C C G A G	36 (0.091)	12 (0.033)	0.0029	0.023	2.92 (1.43-5.62)
C T G A G	14 (0.035)	7 (0.019)	0.387	0.980	1.67 (0.66-4.01)
A C G G G	33 (0.083)	6 (0.017)	1.45×10^{-4}	1.2×10^{-3}	5.08 (2.03-11.22)
C T G G A	7 (0.018)	4 (0.011)	0.897	1.00	0.38-4.32)

[1] -863CA/-857C/T-376GA/-308GA/-238GA haplotype frequency determined by the maximum likelihood method.
[2] Pearson chi-square test.
[3] Pc = corrected *P* as per Bonferroni correction.
[4] OR: odds ratios.
[5] Haplotype frequency.

TNF-α haplotype analysis

Five-locus TNF-α haplotypes were constructed based on the prevalence of individual SNPs, and the LD between them is shown in *table 5*. Of the haplotypes constructed, eight were found to be common, and were included in subsequent analysis. Increased frequencies of CTGGG ($p = 1.7 \times 10^{-4}$), CCGAG ($p = 0.0029$), and ACGGG ($p = 1.45 \times 10^{-4}$) haplotypes, and a reduced frequency of the CCGGG ($p = 4.8 \times 10^{-6}$) haplotype were seen in T1D patients as compared to control subjects *(table 5)*. When the Bonferroni correction was applied, differences were significant for the CTGGG ($Pc = 1.4 \times 10^{-3}$; OR = 2.84; 95% CI = 1.63-4.79), CCGAG (Pc = 0.023; OR = 2.92; 95% CI = 1.43-5.62), and ACGGG ($Pc = 1.2 \times 10^{-3}$; OR = 5.08; 95% CI = 2.03-11.22) haplotypes, which were higher, and the CCGGG haplotype ($Pc = 3.8 \times 10^{-5}$; OR = 0.29; 95% CI = 0.21-0.40) which was lower, among T1D patients, thereby conferring susceptibility to and protection from T1D, respectively.

DISCUSSION

As an autoimmune disease, T1D is accompanied by a state of inflammation, in which heightened levels of pro-inflammation cytokines and their high affinity receptors, including TNF-α, mediate the early onset and progressive destruction of pancreatic β islet cells [17, 18]. The development of hyperglycemia, a hallmark of T1D, appears later in the course of the disease, following months or years of the initiation of the autoimmune destruction of β islet cells [30, 31]. Given the role that TNF-α plays in mediating inflammatory responses and T1D pathogenesis [18, 19], we genotyped known TNF-α polymorphisms in T1D patients and control subjects. As the regulation of the TNF-α gene is genetically determined [20, 21, 32], we focused on six promoter (-1031T/C, -863C/A, -857C/T, -376G/A, -308G/A, -238G/A), and one exon 1 (+488G/A) polymorphisms, previously linked with inflammatory diseases [33]. Our data strongly suggest that -863C/A, -857C/T, -238G/A polymorphisms and certain TNF-α haplotypes are associated with T1D in Tunisians.

TNF-α has been consistently found to play a decisive role in the pathogenesis of several inflammatory diseases, and has been suggested to be a candidate gene for susceptibility to T1D. In our hands, the -308A allele was not associated with T1D. Previous studies linking TNF-α polymorphisms with T1D focused on the -308G/A variant, but with inconsistent findings. Studies on Polish [27], North Indian [13], Egyptian [14], and Koreans [15] reported association between the -308G/A variant and T1D in the population examined, while studies on Belgian [28] and Bahraini [26] subjects showed no such association with T1D. Noble demonstrated that the association of the -308G/A variant with T1D is dependent on the simultaneous presence of specific HLA class II (DRB1, DQB1) alleles [24], while a recent Croatian study suggested limited association of the -308G/A variant with T1D [34]. In a recent meta-analysis, an ethnic contribution to the association of -308G/A variant with T1D was suggested [12]. These discrepancies may be reconciled by differences in sample size [14, 27], ethnic origin [13-15, 27] and, the failure to control for the likely contribution of TNF-α variants. Further analysis is needed to clarify the association of this variant with T1D.

Of the TNF-α variants studied here, the -863C/A, -857C/T, and -238G/A variants were independently associated with the risk of T1D. Our results were in partial agreement with the study of Shbaklo on Lebanese subjects, which, while reporting an association between -857C/T and T1D (as reported here), also claimed an association between the -1031T/C variant and T1D [35]. The study of Nishimura on 165 Japanese T1D patients demonstrated a lack of association between either -1031T/C or -863C/A variants and T1D, especially in young-onset subjects [25]. These inconsistencies most likely reside in differences in ethnic background and in the selection criteria for the T1D cases, further highlighting the need for population-based meta-analyses aimed at determining the contribution of these and other variants to the pathogenesis of T1D.

The 5-locus haplotype analysis revealed extensive diversity in the distribution of the TNF-α haplotypes between T1D patients and controls, with the majority of variation seen in eight common haplotypes. Both susceptible (CCGAG, and ACGGG) and protective (CCGGG) haplotypes were identified after controlling for a number of covariates. Homozygosity for CTGGG ($p = 0.017$), and to a lesser extent ACGGG ($p = 0.044$) haplotypes, was associated with the highest risk of T1D as compared to other haplotype pairs, thereby assigning a dose-effect relationship. While earlier studies reported an association between specific TNF-α microsatellite haplotypes and T1D [28, 36], to the best of our knowledge this is the first report describing an association between specific TNF-α haplotypes and an altered risk of T1D.

In conclusion, our data indicate that TNF-α polymorphisms are associated with the susceptibility to T1D. Several studies, from several independent groups, support the notion of inflammation as a key contributor to T1D [17-19]. Accordingly, heightened TNF-α expression (precipitated by specific TNF-α promoter variants) by autoreactive CD4+ T cells, NK cells, and macrophages, results in increased expression of pro-apoptotic genes [18, 19]. This, in turn, and amplifies the inflammatory responses of islet β cells and induces apoptosis and necrosis. Since TNF-α activates NK cells, and given the role assigned to NK cell in the pathogenesis of T1D [37, 38], this highlights the association of genetic and phenotypic expression of TNF-α with the pathogenesis of T1D. The strengths of this study are in the inclusion of only Tunisian Arabs, thereby minimizing the possibility of ethnic influence on the distribution of the TNF-α variants and thus their association with T1D [29], and in employing haplotype and regression analysis. Our study has some shortcomings, namely that it was relatively underpowered (72.5%), and that we did not correlate genotypic data with TNF-α levels in patients or controls. The possible linkage of the TNF-α variants with other variants in nearby genes in LD, in particular HLA class II [24], cannot be excluded.

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