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# Modulation of Type 1 Diabetes Susceptibility by Tumor Necrosis Factor Alpha −308 G/A and Lymphotoxin Alpha +249 A/G Haplotypes and Lack of Linkage Disequilibrium with Predisposing *DQB1-DRB1* Haplotypes in Bahraini Patients▽

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**Tumor necrosis factor alpha (TNF-α) −308 G/A and lymphotoxin alpha (LTα) +249 A/G single-nucleotide polymorphisms were investigated in 228 type 1 diabetes mellitus (T1DM) patients and 240 controls. Only LTα +249G allele and +249G/+249G genotype frequencies were higher among patients, and no linkage disequilibrium was found between TNF-α/LTα alleles and susceptible/protective *DRB1-DQB1* haplotypes. TNF-α/LTα T1DM-susceptible (−308G/+249G) and protective (−308G/+249A) haplotypes were identified.**

Type 1 (insulin-dependent) diabetes mellitus (T1DM) results from autoimmune destruction of pancreatic β islet cells (1). T1DM susceptibility is determined by environmental, immunologic, and genetic factors (7), including major histocompatibility complex class II genes (2, 11). Other genes lying outside the class II region have also been implicated (7), including the tumor necrosis factor alpha (TNF-α) and lymphotoxin alpha (LTα) genes. TNF-α and LTα are proinflammatory cytokines involved in T1DM pathogenesis (5, 13), since TNF-α enhances cytokine-induced β-cell killing (5, 17) and TNF blockade attenuates T1DM development (13). Their expression is under genetic control (10), with several polymorphisms, including TNF-α −308 G/A and TNF-β +249 A/G single-nucleotide polymorphisms (SNPs), modulating their levels (10).

The association of TNF-α and LTα polymorphisms with T1DM was examined, with inconsistent results. TNF-α −308 G/A SNP was associated with T1DM in some (4, 12, 15), but not all (6, 14), studies. The association of TNF-α −308 G/A with T1DM was suggested by some (6), but not all (12, 15), studies to be due to its carriage with specific DR-DQ haplotypes. This warrants careful assessment of the involvement of TNF alleles in T1DM due to their close proximity to HLA alleles (6, 15). We investigated whether TNF-α −308 G/A and LTα +249 A/G SNPs may be responsible for T1DM susceptibility.

Bahraini study subjects comprised 228 unrelated T1DM patients (109 males and 119 females; mean age, 12.6 ± 8.4 years), diagnosed according to clinical features and laboratory findings. They were receiving insulin for controlling hyperglycemia (with no additional treatment at the time of blood collection), were not obese (mean body mass index = 22.08 ± 7.5), and were free of concomitant complications. Patients with other diabetes forms (type 2, latent autoimmune diabetes in adults, and maturity-onset diabetes of the young, etc.) were excluded. Controls comprised 121 males and 119 females (mean age, 16.3 ± 8.6 years) with normal glucose levels and no family history of T1DM or other autoimmune diseases. All participants were asked to sign a consent form, and all institutional ethics requirements were met.

TNF-α −308 G/A and LTα +249 A/G polymorphisms were determined by PCR-restriction fragment length polymorphism analysis using NcoI digestion (8). Allele frequencies were calculated by the gene-counting method, and both polymorphisms were tested for Hardy-Weinberg equilibrium by using the $\chi^2$ goodness-of-fit test. Allele and genotype frequency differences were tested by Pearson's $\chi^2$ and Fisher's exact tests, and the two-locus association was assessed as described previously (19). Haplotype estimation was done by the expectation maximization method, whereby the sum of probability estimates for all haplotypes equals 1.0. Regression analysis was determined using HPlus 2.5; results are expressed as *P* values, odds ratios (OR), and 95% confidence intervals.

Patients and controls were matched for gender and body mass index, with controls being older than patients ($P = 0.027$). Elevated fasting glucose ($P < 0.001$) and HbA1c ($P < 0.001$) levels were seen in patients. Higher incidences of anti-glutamic acid decarboxylase (100/228 patients versus 0/240 controls; $P < 0.01$), anti-islet cell autoantibodies (83/228 patients versus 0/240 controls; $P < 0.001$), and combined anti-glutamic acid decarboxylase/islet cell autoantibodies (37/228 patients versus 0/240 controls; $P < 0.001$) were seen in patients than in controls.

TNF-α −308 G/A and LTα +249 A/G distribution was in Hardy-Weinberg equilibrium. LTα +249G allele and +249 G/G genotype frequencies were higher among patients, while comparable TNF-α −308 G/A allele and genotype frequencies were seen (Table 1). Linkage disequilibrium (LD) was noted

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TABLE 1. TNF-α −308 G/A and LT-α +249 A/G allele and genotype analysis[d]

| Allele and nucleotide or genotype | Frequency among subjects (mean ± SD)[a] | | $P^b$ | OR[c] |
|---|---|---|---|---|
| | Patients | Controls | | |
| TNF-α −308 G/A | | | | |
| G | 0.85 ± 0.03 | 0.88 ± 0.03 | 0.775 | 0.822 |
| A | 0.15 ± 0.03 | 0.12 ± 0.03 | 0.386 | 1.352 |
| G/G | 0.73 ± 0.03 | 0.79 ± 0.03 | 0.491 | 0.733 |
| G/A | 0.23 ± 0.02 | 0.19 ± 0.02 | 0.546 | 1.344 |
| A/A | 0.03 ± 0.01 | 0.02 ± 0.01 | 0.866 | 1.303 |
| LT-α +249 A/G | | | | |
| A | 0.54 ± 0.04 | 0.69 ± 0.04 | 0.018 | 0.373 |
| G | **0.46 ± 0.04** | **0.31 ± 0.04** | **0.042** | **1.872** |
| A/A | 0.32 ± 0.03 | 0.47 ± 0.03 | 0.061 | 0.530 |
| A/G | 0.45 ± 0.03 | 0.43 ± 0.03 | 0.966 | 1.065 |
| G/G | **0.23 ± 0.02** | **0.10 ± 0.01** | **0.030** | **2.791** |

[a] Subjects comprised 228 T1DM patients and 240 healthy subjects.
[b] Pearson's chi-square test.
[c] Calculated according to the Woolf method.
[d] Data in bold indicate statistically significant differences.

TABLE 2. TNF-α −308 G/A and LT-α +249 A/G haplotype analysis[a]

| TNF-α −308/ LT-α +249 haplotype | Frequency among subjects (mean ± SD) | | $P^b$ | OR[c] (95% confidence interval) |
|---|---|---|---|---|
| | Patients | Controls | | |
| Common haplotypes | | | | |
| G/A | 0.55 ± 0.06 | 0.72 ± 0.04 | <0.001 | 0.47 (0.32–0.69) |
| G/G | 0.31 ± 0.03 | 0.16 ± 0.03 | <0.001 | 2.40 (1.53–3.72) |
| A/G | 0.13 ± 0.04 | 0.10 ± 0.03 | 0.355 | 1.36 (0.77–2.39) |
| Uncommon haplotype A/A | 0.01 | 0.03 | 0.488 | 0.42 (0.10–2.13) |

[a] Determined by the maximum likelihood method.
[b] Fisher's exact test.
[c] Determined according to the Woolf method.

between TNF-α −308G and LTα +249A among patients ($D' = 0.09$; $P = 0.001$) and between TNF-α −308A and LTα +249G among patients ($D' = 0.05$; $P = 0.013$) and controls ($D' = 0.06$; $P = 0.001$). A higher frequency of the −308G/+249G haplotype and a lower frequency of the −308G/+249A TNF-α/LTα haplotype were seen in patients (Table 2). Taking the −308G/+249A TNF-α/LTα haplotype as a reference, multivariate regression analysis confirmed the association of the −308G/+249G TNF-α/LTα haplotype ($P = 0.011$; OR = 1.91) with T1DM, after adjusting for a number of covariates.

TNF-α polymorphisms were previously associated with T1DM and type 2 diabetes. TNF-α −308A and −308A-containing (GA/AA) genotype frequencies were comparable between Bahraini patients and controls, in contrast to studies on non-Caucasian T1DM patients, where an increased frequency of the TNF −308A allele was seen (4, 6, 12). On the other hand, higher LTα +249G allele and homozygous G/G genotype frequencies were seen in Bahraini patients, in agreement with Japanese (14) and U.S. (Caucasian) (15) studies. Collectively, these differences may be reconciled by ethnicity, diabetes type, population stratification, and sample size.

The selective association of LTα, more so than TNF-α, gene variants was demonstrated for some autoimmune diseases (9, 18). Complete LD was seen in our patients between TNF-α −308 and LT-α +249A alleles, which was confirmed by haplotype analysis, with LTα +249G-containing and LTα +249A-containing haplotypes conferring disease susceptibility and protection, respectively. This was reminiscent of a Moroccan study in which a protective effect of a TNF-α/LTα haplotype, independent of LD with HLA class II alleles, was identified (3).

Due its close proximity to HLA genes (7) and because TNF-α −308 G/A polymorphism was in LD with HLA-DR3 in Europeans (16), it was possible that the contributions of TNF-α and LTα polymorphisms to T1DM may by confounded by LD with HLA class II genes, as demonstrated by some (6), but not all (4, 12), studies. It was suggested that disease susceptibility conferred by the TNF-α −308A allele may be due to its carriage by a stronger DR-DQ haplotype (15). Since no LD was found between TNF-α and LTα alleles and high (*DRB1*030101* and *DQB1*0201*)- or low (*DRB1*110101* and *DQB1*030101*)-risk HLA alleles (2), this suggests that the association of the LTα +249 A/G SNP and TNF-α/LTα haplotype with T1DM among Bahraini patients is independent of HLA alleles.

The strengths of this study lie in patient selection (pediatric patients were recruited), thereby excluding the possibility of diabetes misclassification, in the homogeneous ethnicity of the participants (Bahraini Arabs), and in using haplotype analysis, which circumvents the inherent problems of SNP-based association studies. A shortcoming of our study lies in TNF-α and LTα SNPs being genotyped, which raises the possibility that other polymorphisms within TNF-α and LTα genes may play a more significant role in the population studied. It is important to confirm these findings with additional investigations using high-throughput genotyping methods, which would allow screening of large numbers of samples and thus be powered sufficiently to decrease the probability of false-positive associations. Extended haplotype analyses across these and nearby (HLA) genes are needed to elucidate the contributions of TNF genotypes and haplotypes to T1DM susceptibility.

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