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Autoimmune Type 1 Diabetes Genetic Susceptibility Encoded by Human Leukocyte Antigen DRB1 and DQB1 Genes in Tunisia▽

Mouna Stayoussef,[1] Jihen Benmansour,[1] Abdul-Qader Al-Irhayim,[2] Hichem B. Said,[3] Chiheb B. Rayana,[1] Touhami Mahjoub,[1] and Wassim Y. Almawi[2]*

Research Unit of Hematological and Autoimmune Diseases, Faculty of Pharmacy, University of Monastir, Monastir, Tunisia[1]; Department of Endocrinology, CHU Farhat Hached, Sousse, Tunisia[3]; and College of Medicine and Medical Sciences, Arabian Gulf University, Manama, Bahrain[2]

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Human leukocyte antigen (HLA) class II genes contribute to the genetic susceptibility to type 1 diabetes (T1D), and susceptible alleles and haplotypes were implicated in the pathogenesis of T1D. This study investigated the heterogeneity in HLA class II haplotype distribution among Tunisian patients with T1D. This was a retrospective case control study done in Monastir in central Tunisia. The subjects comprised 88 T1D patients and 112 healthy controls. HLA-DRB1 and -DQB1 genotyping was done by PCR-sequence-specific priming. Significant DRB1 and DQB1 allelic differences were seen between T1D patients and controls; these differences comprised *DRB1*030101* and *DQB1*0302*, which were higher in T1D patients than in control subjects, and *DRB1*070101*, *DRB1*110101*, *DQB1*030101*, and *DQB1*060101*, which were lower in T1D patients than in control subjects. In addition, the frequencies of *DRB1*030101-DQB1*0201* and *DRB1*040101-DQB1*0302* were higher in T1D patients than in control subjects, and the frequencies of *DRB1*070101-DQB1*0201* and *DRB1*110101-DQB1*030101* haplotypes were lower in T1D patients than in control subjects. Multiple logistic regression analysis revealed the positive association of *DRB1*030101-DQB1*0201* and *DRB1*040101-DQB1*0302* and the negative association of only *DRB1*070101-DQB1*0201* haplotypes with T1D. Furthermore, a significantly increased prevalence of *DRB1*030101-DQB1*0201* homozygotes was seen for T1D subjects than for control subjects. Our results confirm the association of specific HLA-DR and -DQ alleles and haplotypes with T1D in Tunisians. The identification of similar and unique haplotypes in Tunisians compared to other Caucasians highlights the need for evaluating the contribution of HLA class II to the genetic susceptibility to T1D with regard to haplotype usage and also to ethnic origin and racial background.

Type 1 (insulin-dependent) diabetes (T1D) is the most prevalent form of diabetes in children and young adults (12, 17) and results from autoimmune $CD4^+$ and $CD8^+$ T-cell-directed destruction of insulin-producing pancreatic ß islet cells, leading to irreversible hyperglycemia and related complications (4, 22). In addition to environmental factors, there is a strong genetic component to T1D pathogenesis, of which the human leukocyte antigen (HLA) locus, in particular the class II region (DR and DQ), account for 40 to 50% of T1D familial clustering (13, 30). This was evidenced by the enrichment of DR3, DR4, DQ2, and DQ8, and the lower prevalence of DR15 or DQ6.2 alleles among T1D patients, thereby assigning a susceptible or protective role for these alleles in T1D pathogenesis, respectively (3, 16, 21).

The fact that not all carriers of a specific high-risk DR or DQ variant develop the disease and the strong linkage disequilibrium between select DRB1 and DQB1 alleles (28) indicate that the pathogenesis of T1D results from the complex interaction between several genes within the class II region, in which specific DRB1-DQB1 haplotypes contribute to disease susceptibility. Accordingly, the enrichment or decreased prevalence of select DRB1-DQB1 haplotypes in T1D patients imparts disease susceptibility or protection, respectively (3, 18, 24). This susceptibility or protection effect disappears when a different DRB1 or DQB1 allele replaces the specific allele in the haplotype (29). The contribution of specific HLA haplotypes toward T1D susceptibility depends on the ethnic/racial background (26), which was highlighted by the positive association of *DRB1*030101-DQB1*0201* and *DRB1*040101-DQB1*0302* haplotypes with T1D among Caucasians (3, 16) compared to *DRB1*0405-DQB1*0401* and *DRB1*0901-DQB1*0303* haplotypes and T1D in Japanese (18), while *DRB1*1501-DQB1*0602* appeared to be protective of T1D in all populations (3, 16, 18). This indicates that association of a specific class II allele and DRB1-DQB1 haplotype with T1D must be evaluated in the context of the specific ethnic/racial background (26).

We previously reported an association between HLA DRB1 and DQB1 alleles and haplotypes in Tunisian T1D patients ($n = 50$) and control subjects ($n = 50$) and identified two susceptible haplotypes (*DRB1*030101-DQB1*0201* and *DRB1*040101-DQB1*0302*), but no protective haplotypes (27). Using haplotype estimation and regression analysis, here, we extend our investigation of HLA class II and T1D risk on a large sample size by confirming the association of these haplotypes and identified an additional T1D-protective haplotype.

MATERIALS AND METHODS

Subjects. Study subjects comprised 88 unrelated T1D patients (44 males and 44 females; age [mean ± standard error], 16.4 ± 7.7 years). The diagnosis of T1D was according to both clinical features and laboratory data. All T1D patients

* Corresponding author. Mailing address: Department of Medical Biochemistry, College of Medicine and Medical Sciences, Arabian Gulf University, P.O. Box 22979, Manama, Bahrain. Phone: 973-39717118. Fax: 973-17271090. E-mail: wassim@agu.edu.bh.

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TABLE 1. HLA-DRB1* allele distribution

*DRB1** allele	T1D patients (n = 88)		Controls (n = 112)		P^a	Pc^b	OR
	Mean allele frequency	SE	Mean allele frequency	SE			
010101	0.0455	0.0157	0.0804	0.0182	0.222	3.559	0.559
030101	**0.3636**	**0.0363**	**0.1473**	**0.0237**	$\mathbf{3.6 \times 10^{-4}}$	**0.006**	**2.866**
030201	0.0000	0.0000	0.0134	0.0077	0.122	1.950	0.000
0317	0.0227	0.0112	0.0179	0.0088	0.951	15.209	0.953
040101	**0.2386**	**0.0321**	**0.1161**	**0.0214**	$\mathbf{7.5 \times 10^{-3}}$	**0.120**	**2.298**
070101	**0.0682**	**0.0190**	**0.2143**	**0.0274**	$\mathbf{1.8 \times 10^{-4}}$	**0.003**	**0.257**
080101	0.0170	0.0098	0.0223	0.0099	0.705	11.287	0.755
090102	**0.0284**	**0.0125**	**0.0000**	**0.0000**	**0.023**	**0.362**	**NA**[c]
100101	**0.0227**	**0.0112**	**0.0625**	**0.0162**	**0.051**	**0.816**	**0.333**
110101	**0.0284**	**0.0125**	**0.1071**	**0.0207**	$\mathbf{1.7 \times 10^{-3}}$	**0.027**	**0.221**
120101	0.0170	0.0098	0.0045	0.0045	0.207	3.313	3.918
130101	0.0966	0.0223	0.1250	0.0221	0.248	3.967	0.667
1325	0.0000	0.0000	0.0045	0.0045	1.000	16.000	0.000
140101	0.0000	0.0000	0.0134	0.0077	0.122	1.950	0.000
150101	0.0227	0.0112	0.0625	0.0162	0.075	1.208	0.363
160101	0.0284	0.0125	0.0089	0.0063	0.137	2.187	3.313

[a] P values were determined by Fisher's exact test. Boldface values indicate that there were significant differences between the mean allele frequencies for the patients with T1D and controls.
[b] Pc, corrected P values for the number of alleles tested, calculated using the Bonferonni method.
[c] NA, not available.

were ketosis prone, lacked endogenous insulin secretion, and needed insulin for controlling hyperglycemia. T1D patients were not obese, were free of any concomitant complication, and were not receiving additional treatment at the time of blood collection. Patients with other forms of diabetes were excluded. Control subjects consisted of 112 university students and healthy children (65 males and 47 females; age [mean ± standard error], 28.2 ± 5.8 years), who had normal glucose tolerance and no family history of T1D or other autoimmune diseases. All patients and control subjects were Tunisian Arabs, were from central Tunisia, and were asked to sign a consent form according to the study protocol, and all institutional ethics requirements were met.

HLA-DRB1 and -DQB1 genotyping. HLA-DRB1 and -DQB1 alleles were analyzed using the PCR-sequence-specific priming technique, using the Micro SSP generic HLA class II (DRB/DQB) DNA typing kit (lot *05A*), according to the manufacturer's specifications (One Lambda, Thousand Oaks, CA). PCR products were analyzed on ethidium bromide-stained agarose gels. HLA allele nomenclature was as previously reported (23). In total, 16 DRB1 and 7 DQB1 alleles were tested.

Data analysis. Allele frequencies were determined by the gene counting method (31), using the HLAStat 2000 software, which also computed the P values (Fisher's exact probability test) and odds ratios (OR). The frequencies of the most probable HLA DRB1-DQB1 haplotypes were determined by the maximum-likelihood method, using the Arlequin (v. 2.000) population genetics data analysis software (25). To minimize the possibility of spurious association or chance finding, P values were corrected (Pc) for the number of different alleles tested (n) using the Bonferroni inequality method [$Pc = 1 - (1 - P)^n$] (7) and P values were corrected for the different haplotypes analyzed using the Sidak correction factor [$Pc = 1 - (1 - P)^{1/n}$]. These tests are used when several dependent or independent tests are performed simultaneously, where the individual P value may not be appropriate for all comparisons. Additional statistical analysis was performed with SPSS version 14.0 for Windows statistical package (SPSS Inc., Chicago, IL).

RESULTS

HLA-DRB1 and HLA-DQB1 allele frequencies. Significant DRB1 allelic differences were seen between T1D patients and controls, with 6 of 16 loci being significantly different ($P < 0.05$). When Bonferroni's correction was applied, differences were significant for only three loci, which comprised *DRB1*030101* ($Pc = 0.006$), which was higher among T1D patients, and *DRB1*070101* ($Pc = 0.003$) and *DRB1*110101* ($Pc = 0.027$), which were higher in control subjects (Table 1). Similarly, significant DQB1 allelic differences were seen at the DQB1 locus, three of seven loci being significantly different even after Bonferroni's correction. These comprised *DQB1*0302* ($Pc = 0.012$), which was higher among T1D patients, and *DQB1*030101* ($Pc = 0.007$) and *DQB1*060101* ($Pc = 0.041$), which were higher among control subjects (Table 2).

TABLE 2. HLA-DQB1* allele distribution

*DQB1** allele	Patients (n = 88)		Controls (n = 112)		P^a	Pc^b	OR
	Mean allele frequency	SE	Mean allele frequency	SE			
0201	0.4261	0.0373	0.2902	0.0303	0.054	0.377	1.750
030101	0.0966	0.0223	0.2009	0.0268	0.001	0.007	0.308
0302	0.2159	0.0310	0.0938	0.0195	0.002	0.012	2.760
030302	0.0739	0.0197	0.0536	0.0150	0.724	5.068	1.177
0401	0.0227	0.0112	0.0625	0.0162	0.075	0.528	0.363
050101	0.0852	0.0210	0.1205	0.0218	0.135	0.943	0.573
060101	0.0795	0.0204	0.1786	0.0256	0.006	0.041	0.384

[a] P values were determined by Fisher's exact test.
[b] Pc, corrected P values for the number of alleles tested, calculated using the Bonferonni method.

TABLE 3. *DRB1*-DQB1** haplotype distribution

DRB1-DQB1** haplotype[a]	Haplotype frequency		*P*[c]	*Pc*[c]	OR (95% CI)
	Patient[b]	Control[b]			
*DRB1*010101-DQB1*050101*	0.035	0.060	0.378	0.978	
*DRB1*030101-DQB1*0201*	0.287	0.065	<0.001	<0.001	5.53 (2.93–9.93)
*DRB1*040101-DQB1*0302*	0.184	0.073	0.001	0.010	2.89 (1.51–5.33)
*DRB1*070101-DQB1*0201*	0.068	0.177	0.002	0.015	0.34 (0.18–0.68)
*DRB1*100101-DQB1*030101*	0.017	0.026	0.755	1.000	
*DRB1*110101-DQB1*030101*	0.023	0.097	0.005	0.036	0.21 (0.08–0.66)
*DRB1*130101-DQB1*060101*	0.051	0.093	0.157	0.745	
*DRB1*150101-DQB1*060101*	0.023	0.055	0.192	0.818	

[a] *DRB1** and *DQB1** alleles were assessed by PCR-sequence-specific priming, and haplotype frequencies were determined by the maximum-likelihood method.
[b] Subjects comprised 88 T1D patients and 112 control subjects.
[c] *P* values and *Pc* values were determined by Fisher's exact test.

Frequencies of DRB1-DQB1 haplotypes. Of the eight frequent haplotypes identified, the frequencies of *DRB1*030101-DQB1*0201* ($Pc < 0.001$), and *DRB1*040101-DQB1*0302* ($Pc = 0.010$) were higher among T1D patients, thereby conferring T1D susceptibility to these haplotypes (Table 3). In addition, the frequencies of *DRB1*070101-DQB1*0201* ($Pc = 0.015$) and *DRB1*110101-DQB1*030101* ($Pc = 0.036$) were lower in T1D patients than in control subjects, thus assigning a disease-protective nature to these haplotypes (Table 3).

Regression analysis. The contribution of specific HLA DRB1-DQB1 to T1D was analyzed by multiple logistic regression analysis. Logistic regression analysis confirmed that *DRB1*030101-DQB1*0201* (OR, 3.88; 95% confidence interval [95% CI], 1.88 to 8.02) and *DRB1*040101-DQB1*0302* (OR, 2.91; 95% CI, 1.35 to 6.23) were positively associated, while *DRB1*070101-DQB1*0201* (OR, 0.37; 95% CI, 0.16 to 0.85) was negatively associated with T1D (Table 4). The initial negative association of *DRB1*110101-DQB1*030101* with T1D was rejected according to the model employed.

DRB1-DQB1 genotype distribution. We assessed the contribution of the major HLA haplotypes identified to the presence of T1D by comparing the frequencies of the T1D-susceptible haplotype (*DRB1*030101-DQB1*0201* and *DRB1*040101-DQB1*0302*) and T1D-protective haplotype (*DRB1*070101-DQB1*0201* and *DRB1*110101-DQB1*030101*) homozygotes and heterozygotes in T1D patients and in controls. Similarly, the frequencies of heterozygotes and homozygotes with the DR4 haplotype (*DRB1*0405-DQB1*0401*) were also tested in T1D patients and in control subjects. Significant DRB1-DQB1 genotype differences were seen between T1D patients and controls. *DRB1*030101-DQB1*0201* homozygotes ($P = 4.2 \times 10^{-5}$) and heterozygotes ($P = 0.015$) and *DRB1*040101-DQB1*0302* homozygotes ($P = 0.017$) were more frequent in T1D patients than in control subjects (Table 5). After the *P* values were adjusted, differences were significant for only *DRB1*030101-DQB1*0201* homozygotes ($P = 2.5 \times 10^{-4}$), which was higher among T1D patients (Table 5).

TABLE 4. Multinomial regression analysis

DRB1-DQB1** haplotype	*P*	OR	95% CI
*DRB1*030101-DQB1*0201*	2.5×10^{-4}	3.881	1.879–8.019
*DRB1*040101-DQB1*0302*	0.006	2.905	1.354–6.231
*DRB1*070101-DQB1*0201*	0.020	0.368	0.159–0.853
*DRB1*110101-DQB1*030101*	0.077	0.370	0.123–1.113

DISCUSSION

Results obtained demonstrated that the contribution of HLA haplotypes to T1D genetic susceptibility among Tunisians depends on specific HLA class II haplotypes. The *DRB1*030101-DQB1*0201* haplotype fitted a recessive model, since it confers strong T1D susceptibility when present in a homozygous state ($Pc = 2.5 \times 10^{-4}$; OR = 43.79), rather than in a heterozygous state ($Pc = 0.089$; OR = 5.19). The high T1D susceptibility conferred by both *DRB1*030101-DQB1*0201* and *DRB1*040101-DQB1*0302* haplotypes was reminiscent of previous studies of Caucasians (3, 13, 16, 19), but not non-Caucasians (12), supporting the notion of Caucasian T1D susceptibility haplotypes.

*DQB1*0302* was strongly associated with, while *DQB1*030101* was largely protective of T1D. Similar associations were reported for northern Europe (8, 14–16), but not southern Europe (15, 24) or Mediterranean countries (3), in which *DQB1*0201* was reported as the major DQB1 susceptible allele. This lack of association of *DQB1*0201* with T1D in Tunisians was supported by the finding that *DQB1*0201* was linked with T1D susceptibility when present with *DRB1*030101* but was negatively associated with T1D when present with *DRB1*070101* in a haplotype. Thus, it appears that *DQB1*0201* did not play a significant role in T1D pathogenesis and that the disease protection or susceptibility may be explained the presence of *DQB1*0201* haplotypes with protective or susceptible *DRB1* alleles, respectively, as was also suggested elsewhere (10).

*DRB1*030101-DQB1*0201* and *DRB1*040101-DQB1*0302* were strongly associated with, while *DRB1*070101-DQB1*0201* was protective of T1D, further supporting the notion that *DRB1*030101-DQB1*0201* on its own is a major T1D susceptibility haplotype among Caucasians (3, 13, 19). Our findings were reminiscent of earlier studies of Tunisians, which showed that DR3 and DR4 (1, 5) and *DRB1*03-DQB1*0201* and *DRB1*04-DQB1*0302* haplotypes (1) were strongly associated with T1D. The notable difference was the identification of *DRB1*070101-DQB1*0201* as the T1D-protective haplotype in our study, compared to *DRB1*1501-DQB1*0602* reported by Abid Kamoun (1). While explanation for these apparent

TABLE 5. Genotypic combination of DRB1-DQB1 haplotypes

DRB1-DQB1*/DRB1*-DQB1** combination	% Patients[a]	% Controls[a]	*P*[b]	*Pc*[c]	OR
*DRB1*030101-DQB1*0201/DRB1*030101-DQB1*0201*	14 (15.9)	0 (0.0)	4.2×10^{-5}	2.5×10^{-4}	43.79
*DRB1*040101-DQB1*0302/DRB1*040101-DQB1*0302*	6 (6.8)	0 (0.0)	0.017	0.097	17.73
*DRB1*030101-DQB1*0201/DRB1*040101-DQB1*0302*	11 (12.5)	3 (2.7)	0.015	0.089	5.19
*DRB1*070101-DQB1*0201/DRB1*070101-DQB1*0201*	1 (1.1)	6 (5.4)	0.221	0.777	
*DRB1*110101-DQB1*030101/DRB1*110101-DQB1*030101*	2 (2.3)	3 (2.7)	0.784	1.000	
*DRB1*070101-DQB1*0201/DRB1*110101-DQB1*030101*	0 (0.0)	2 (1.8)	0.586	1.000	

[a] The percentage of total within group is shown in parentheses.
[b] *P* values were determined by Fisher's exact test.
[c] *P* values adjusted using the Sidak correction factor.

discrepancies remain speculative at this stage, it is likely due to sample size differences, selection of study subjects, and the failure to control for potential covariates by earlier studies (1, 5).

The identification of *DRB1*030101-DQB1*0201* and *DRB1*040101-DQB1*0302* as T1D-susceptible haplotypes and of *DRB1*070101-DQB1*0201* and *DRB1*110101-DQB1*030101* as T1D-protective haplotypes was comparable to previous results with Caucasians (3, 13, 19, 21). Of these haplotypes, regression analysis rejected *DRB1*110101-DQB1*030101* as a T1D-protective haplotype, and its presence in a homozygous or heterozygous state did not impart any T1D protection aspect according to the model employed. Previous identification of low-risk or protective haplotypes, including *DRB1*110101-DQB1*030101*, may imply protection, or may be the consequence of reduction in its frequency in T1D patients brought about by corresponding increase in frequency of susceptibility haplotypes (*DRB1*040101-DQB1*0302* and *DRB1*030101-DQB1*0201*), as was suggested elsewhere (10). Collectively, this supports the notion of intricate interplay between individual DRB1 and DQB1 loci in determining susceptibility to T1D.

HLA class II DR and DQ complex bind antigen fragments and direct the presentation of antigens to T cells. The presence (or absence) of certain residues within the peptide-binding sites of the DR-DQ complex was suggested to dictate the predisposition to or protection from disease, including T1D (10, 20). By binding ß-cell-specific peptides in the context of peptide-major histocompatibility complex complex, specific class II haplotypes are involved in activation and later expansion of autoreactive T cells (2, 9, 11). As such, the strong association of *DRB1*030101-DQB1*0201* and *DRB1*040101-DQB1*0302* and the negative association of *DRB1*070101-DQB1*0201* with T1D may be explained by differences in affinity to (autoantigenic) peptide fragments presented by each haplotype. This may involve fitting of these peptide fragments within the respective haplotype binding grooves and would be useful in the screening of additional autoantigens linked with diabetes and in the identification of specific epitopes likely to interact with diabetogenic autoreactive T cells (2, 6).

The identification of positive and negative association of specific HLA class II haplotype to T1D pathogenesis may have important clinical implications, likely by allowing identification of at-risk individuals, and thus early intervention. However, despite the strength of the association observed, our study has some limitations, namely, that it was limited to the HLA DRB and DQB regions, and thus did not allow for examination of the possible association of additional HLA loci, or other genes in linkage disequilibrium with HLA alleles, with T1D. Our results highlight the significance of analyzing haplotypes and genotypic combinations, as opposed to single alleles, in assigning T1D genetic susceptibility. Accordingly, a specific haplotype may modulate the susceptible/protective nature of another haplotype within a particular genotype combination.

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