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Linkage disequilibrium with predisposing DR3 haplotypes accounts for apparent effects of TNF and LTA polymorphisms on T1D susceptibility

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Abstract

Tumor necrosis factor (TNF) and lymphotoxin alpha (LTA) are immunomodulators that have been hypothesized to contribute to susceptibility to type 1 diabetes (T1D). Several polymorphisms in the TNF and LTA loci have been extensively studied for T1D association, with conflicting reports. In this study, we examined two TNF variants and one LTA variant for T1D association in 283 Caucasian, multiplex T1D families, for which complete HLA genotyping data are available. Initially, association with T1D was seen for LTA A1069G (intron A) ($p = 0.011$) {rs909253} and TNF G(-308)A ($p < 1 \times 10^{-5}$) {rs1800629}, but no association was observed for TNF G(-238)A {rs361525}. After adjusting the data for linkage disequilibrium (LD) with DRB1-DQB1 haplotypes, however, only TNF G(-238)A showed significant association with T1D ($p < 0.006$). When HLA-DR3 haplotypes were examined, the A allele of TNF G(-238)A was significantly overtransmitted to affected offspring ($p < 0.009$). Including HLA-B data in the analysis revealed that TNF (-238)A is present exclusively on DR3 haplotypes that also carry HLA-B18. Transmission proportion of B18-DR3 haplotypes did not differ between those with TNF (-238)A and those with TNF (-238)G. Thus, variation at TNF does not affect the T1D risk for B18-DR3 haplotypes, and the apparent association of TNF(-238)A with T1D may simply reflect its presence on a high-risk haplotype.

Keywords

TNF; Polymorphisms; HLA; Type 1 diabetes; Linkage disequilibrium

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of insulin producing beta cells in the pancreas (1). Although multiple genes have been implicated, the HLA region genes encoding the DR and DQ proteins are well established as the major T1D susceptibility loci. Other HLA loci contribute to T1D susceptibility, including an additional HLA class II locus, DPB1, and the HLA class I loci A, B, and C [1–3]. The region that lies between the HLA class II and HLA class I regions, sometimes referred to as “class III,” or the

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“central MHC,” contains a number of genes related to inflammatory and immune responses, including tumor necrosis factor (TNF) and lymphotoxin alpha (LTA), which has also been referred to as TNF- β . TNF and LTA are closely spaced and tandemly arranged on chromosome 6p21. Their protein products have related functions and both bind the same receptor. Associations of polymorphisms in the TNF and LTA loci have been reported for a number of diseases, including, but not limited to, asthma [4], sarcoidosis [5], rheumatoid arthritis [6], Inflammatory Bowel Disease [7], and T1D (see below).

TNF is a major proinflammatory cytokine implicated in islet beta-cell destruction, which results in T1D [8]. Results in animal models [9] indicate that CD4⁺ T lymphocytes, a major source of IFN- γ , act in collaboration with macrophages, as a major source of TNF, to induce pancreatic beta cell death. LTA is a cytokine produced by lymphocytes that mediates a large variety of inflammatory, immunostimulatory, and antiviral responses. LTA is also involved in the formation of secondary lymphoid organs during development and plays a role in apoptosis. Variations at several TNF and LTA polymorphisms have been reported to correlate with expression levels [7,10], although the functional significance of these reports is somewhat controversial [11].

Genetic associations of TNF and LTA polymorphisms with T1D have been extensively investigated. Because of their proximity to and linkage disequilibrium (LD) with the HLA class II genes, polymorphisms in the TNF and LTA loci frequently appear to be associated with T1D. For example, an apparent genetic association of TNF with T1D in patients with severe disease was reported in a Chilean study [12]. However, adjustment of the data for LD with HLA class II alleles can change the apparent association of these polymorphisms with T1D. Examples of this include a report of a study of Finnish patients in which no association between LTA restriction fragment length polymorphisms (RFLPs) and T1D was seen after adjustment for DRB1-DQB1 genotype [13]. In a Japanese population [14], after adjustment for class II haplotypes, no significant association of LTA (intron 1 “NcoI”) or TNF (–1037, –857) single nucleotide polymorphisms (SNPs) with T1D was detected. On the other hand, Bouqbis and coworkers [15] reported that TNF (–307) -LTA (+252) haplotypes defined protective HLA class II haplotypes in a Moroccan sample. Separating T1D risk conferred by HLA alleles and that conferred by TNF or LTA alleles can be difficult but is necessary to determine whether or not apparent associations with T1D are real.

In this study, we examined a collection of 283 Caucasian, multiplex T1D families from the Human Biological Data Interchange (HBDI) repository, a substantially larger sample set than those utilized in previous reports of T1D association with polymorphisms in TNF and LTA loci. In addition to its size, this collection of family-based samples has the added advantage of having been completely characterized for the classical HLA-class II and HLA-class I genes in our laboratory. We utilized a genotyping assay that was originally designed to test a set of inflammation-associated polymorphisms (see Methods) and analyzed the genotyping data from the following two TNF SNPs and one LTA SNP: TNF G(–308)A, TNF G(–238)A, and LTA A1069G (intron A). These polymorphisms have been extensively studied in other populations not only for disease association but also for TNF expression levels. We examined the transmission proportions of these polymorphisms first in the total sample, then with data adjusted for LD with HLA DR- and DQ-encoding genes, and, finally, in the following individual, highly-predisposing haplotypes: DRB1*0301-DQB1*02, DRB1*0401-DQB1*0302, DRB1*0404-DQB1*0302, and DRB1*0405-DQB1*0302 or *02.

SUBJECTS, MATERIALS, AND METHODS

Subjects

DNA samples from 283 Caucasian, multiplex families were obtained from the collection of the Human Biological Data Interchange (HBDI, Philadelphia, PA). This HBDI is a repository for cell lines derived from type 1 diabetes (T1D) families and was established in part for the purpose of mapping T1D-associated genes by linkage analysis. Samples may be purchased by qualified investigators and are supplied as purified DNA. The samples are identified with a repository number that corresponds to a family code and member designation. Investigators are not provided with identities of individuals whose DNA is included in the collection.

Additional information can be found on the HBDI website

(<http://www.ndriresource.org/html/hbdi.htm>). The HBDI operates under the guidance of the Internal Review Board of the University of Pennsylvania. The HBDI families included in this report are nuclear families with at least two affected siblings (multiplex). Most have unaffected parents. A total of 709 patients (46.5% female) were genotyped for this study. The mean age at onset of T1D was 11.25 years (SD=7.6).

Genotyping methods

HLA and SNP genotyping data were generated using the linear array method, a PCR/Sequence-Specific Oligonucleotide Probe (SSOP) method that has been described elsewhere [16].

Briefly, unlabelled oligonucleotide probes, corresponding to known sequence motifs in the locus or loci of interest, are immobilized on a backed nylon membrane. Biotinylated PCR primers are used to amplify the locus/loci of interest. The amplification product is then hybridized to the oligonucleotide array, and binding is detected by a colorimetric detection system. Genotypes are deduced from the probe binding pattern. The following SNPs were examined in this study: LTA A1069G (intron A) {rs909253}, TNF G(-308)A {rs1800629}, TNF (-238) {rs361525}. The TNF SNP data were generated utilizing a linear array panel of inflammation-related SNPs. This panel has been described elsewhere [17].

Statistical methods

A transmission disequilibrium test (TDT); [18] was carried out on DRB1-DQB1 heterozygous parents carrying at least one of the following haplotypes DRB1*0301-DQB1*02, DRB1*0401-DQB1*0302, DRB1*0404-DQB1*0302, or DRB1*0405-DQB1*0302 or *02.

Under the null hypothesis that the loci encoding DR and DQ are the only T1D-associated loci in the HLA region, the expectation is that transmission of alleles at other polymorphic loci to affected offspring should be random. Under this hypothesis, the transmission proportions for the alleles of the tested polymorphisms should be constant across all HLA haplotypes, regardless of the T1D susceptibility attributable to the HLA haplotype. Deviations from such random expectation were assessed using a chi-square test. In cases where the total sum in the contingency table was <200, a Fisher's exact test was employed. Not transmitted = TNF SNP allele frequency among DR3 or DR4 haplotypes not transmitted to affected offspring from heterozygote parents. Transmitted = allele frequency among DR3 or DR4 haplotypes transmitted to affected offspring. % trans = percent times that a given haplotype was transmitted to affected offspring = Transmitted/(Not Transmitted + Transmitted).

RESULTS

Table 1 shows the transmission proportions for the LTA A1069G, TNF G(-308)A, and TNF G(-238)A SNPs in the total set of 283 families. The LTA A1069G, and TNF- α G(-308)A SNPs appear to have strong positive association with T1D ($p < 0.011$ and $p < 1 \times 10^{-5}$, respectively), while the TNF G(-238)A SNP shows no apparent association. Notably, the two SNPs that

showed significant association with T1D in the initial analysis were also found to be in strong linkage disequilibrium (LD) with DRB1*0301-DQB1*02 haplotypes (referred to as “DR3 haplotypes”), which are highly-predisposing for T1D, while the SNP that showed no apparent association also showed no LD with DR3 haplotypes. DR3 is common in T1D patients. Haplotype frequency of DR3 was 31.4% for affected sibs but only 9.5% for the affected family based control (AFBAC) haplotypes [19], and 328 of 566 patients (58.0%), representing 185 families, had at least one DR3 haplotype (not shown). Table 2 shows the transmission proportions for the three polymorphisms after the expected values have been adjusted to account for the LD between the TNF loci and HLA class II haplotypes. Therefore, expected values are no longer 50% for each allele of the biallelic SNPs, but, rather, are estimated based on the LD values observed between the TNF SNP alleles and HLA class II haplotypes in the AFBAC haplotypes [19] in this data set. The results in Table 2 are in striking contrast to those in Table 1. After adjustment for LD, the associations observed in Table 1 for the LTA A1069G and TNF G(-308)A SNPs lose significance, while the TNF G(-238)A SNP is now positively associated with T1D ($p < 0.006$).

Because the haplotypes DR3 and DR4 are most strongly associated with T1D in Caucasians, we examined the transmission proportion of the TNF SNPs on those haplotypes. For DR3 haplotypes, all of which include the alleles DRB1*0301 and DQB1*02, Table 3 shows that the A allele of the TNF G(-238)A SNP is significantly overtransmitted (81.0%; $p < 0.009$). This demonstrates risk heterogeneity of DR3 haplotypes and is consistent with the hypothesis that this polymorphism, or another locus in LD with it, may affect T1D susceptibility independently of HLA class II loci. However, risk heterogeneity of DR3 haplotypes has been previously demonstrated, with those DR3 haplotypes that carry B18 alleles having higher T1D risk than those DR3 haplotypes that carry B8 alleles [20]. We found that the putative predisposing allele “A” of the TNF G(-238)A SNP is found on B18-DR3 haplotypes in this data set but is absent from B8-DR3 haplotypes. We examined the transmission proportion of all three TNF SNPs on B18-DR3 and on B8-DR3 haplotypes (Table 4). No significant transmission distortion was present on B18-DR3 haplotypes for any of the three TNF polymorphisms (Table 4). These results are consistent with the notion that the TNF- α (-238)A may simply be a marker for a highly predisposing B18-DR3 haplotype rather than having a susceptibility effect that can be attributed to the TNF polymorphism itself. This hypothesis is supported by the fact that the transmission proportion of B18-DR3 haplotypes is nearly identical between haplotypes carrying TNF (-238)A and those carrying TNF (-238)G (Table 4).

B8-DR3 haplotypes that include the A allele of the LTA A1069G SNP were undertransmitted with borderline significance (Table 4, $p < 0.05$); however, the numbers are small, and the p value was not adjusted for multiple testing. Neither of the other TNF polymorphisms exhibited transmission distortion on B8-DR3 haplotypes. Undertransmission of the A allele of the LTA A1069G SNP was also observed for DR4 haplotypes that carry the DRB1*0405 allele, but, again the result is based on small numbers (9 not transmitted vs. 5 transmitted, $p < 0.05$, data not shown). No other DR4 haplotypes had significant transmission distortion of any of the three SNPs tested (data not shown). While the observation that the same LTA allele appears protective on two different DR haplotypes is notable, the hypothesis that the allele is protective (or is in LD with a protective allele at another locus) requires further testing in a much larger sample set.

DISCUSSION

The data reported here illustrate the need for caution in interpretation of T1D association studies for SNPs in the HLA region. The apparent T1D association of each of the LTA and TNF SNPs examined in this study changes following adjustment for LD with the DRB1-DQB1 haplotypes. A similar change in apparent effect on T1D susceptibility was previously reported in our

analysis of HLA-A genotyping data from the same set of families [1]. Before adjustment for LD with DRB1-DQB1 haplotypes, the allele A*0101 appeared significantly predisposing for T1D ($p<0.007$), but after adjustment, A*0101 appeared significantly protective ($p<0.007$). The A*0101 allele on that haplotype is clearly not a contributing factor to genetic risk for T1D and, in fact, may contribute to the observation that A1-B8-DR3 haplotypes are less predisposing than are B18-DR3 haplotypes. The data presented here show that the TNF (-238)A allele is present on B18-DR3 haplotypes and not on B8-DR3 haplotypes. Although the hypothesis could be made that the TNF (-238)A allele contributes to the higher T1D risk of B18-DR3 vs. B8-DR3 haplotypes, the similar transmission proportions of B18-DR3 haplotypes with and without the TNF- α (-238)A allele argue against this. In summary, these data show no evidence of a causal role in T1D susceptibility for polymorphisms in the LTA and TNF loci.

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ABBREVIATIONS

TNF	tumor necrosis factor
SNP	single nucleotide polymorphism
HLA	Human Leukocyte Antigen
T1D	Type 1 Diabetes
LD	Linkage Disequilibrium

Table 1

Transmission disequilibrium test of TNF and LTA SNPs and linkage disequilibrium with DRB1*0301-DQB1*02 haplotypes in 283 multiplex T1D families.

SNP	Allele	Trans ⁽¹⁾	Trans % of "A"	McNemar Chi- sq TDT	P< for TDT test	D' with DR3 ⁽²⁾	P< for D'
LTA A1069G (intron A)	A	256				−0.568	
	G	314	44.7%	6.49	0.011	0.568	1 x 10 ^{−7}
TNF G (−308)A	A	242				0.651	
	G	154	61.1%	19.56	1x 10^{−5}	−0.651	1 x 10 ^{−22}
TNF G (−238)A	A	76				0.027	
	G	69	51.4%	0.11	0.737	−0.027	n.s.

(1) Only informative transmissions are shown.

(2) D' = normalized linkage disequilibrium coefficient

Table 2

Association of TNF and LTA SNPs with T1D after adjusting for linkage disequilibrium with DRB1-DQB1 haplotypes in 283 multiplex families.

SNP	Allele	AFBAC frequency ⁽¹⁾	Observed T1D frequency	exp T1D freq given LD ⁽²⁾	P<
LTA A1069G (intron A)	A	68.7%	59.8%	59.3%	0.793
	G	31.3%	40.2%	40.7%	
TNF G(-308)A	A	15.3%	25.9%	27.6%	0.351
	G	84.7%	74.1%	72.4%	
TNF G(-238)A	A	6.5%	6.3%	3.7%	0.006
	G	93.5%	93.8%	96.3%	

(1) AFBAC = affected family based controls [19]

(2) under the null hypothesis that differences in SNP allele frequencies between T1D and controls are due exclusively to linkage disequilibrium with DRB1-DQB1 the expected allele frequency in T1D cases is given by $\exp[\text{marker}(i) \text{ T1D}] = \text{marker}(i) \text{ controls} + \sum D_{ij} \text{DR}(j) \text{ T1D} / \text{DR}(j) \text{ controls}$ where $\text{marker}(i) \text{ T1D}$ denotes the frequency of allele i at the marker under study among cases and controls respectively, D_{ij} is the pairwise gametic disequilibrium coefficient among controls between the j th haplotype at DRB1-DQB1 and the i th allele at the marker, and $\text{DR}(j) \text{ T1D}$, $\text{DR}(j) \text{ controls}$ denote the frequencies in cases and controls of the j th DRB1-DQB1 haplotype. The sum is over all DRB1-DQB1 haplotypes.

Table 3

Proportion of DR3 haplotypes transmitted to T1D affected offspring carrying individual alleles of TNF and LTA SNPs. The frequencies of not transmitted and transmitted haplotypes among specific DR-DQ haplotypes (counts in parentheses) are shown.

DRB1 DQB1	LTA A1069G	TNF G (-238)A	TNF G (-308)A	Not Transmitted	Transmitted	% trans	P<
0301 02	A			0.274 (45)	0.337 (120)	72.7%	n.s.
	G			0.726 (119)	0.663 (236)	66.5%	
0301 02		A		0.091 (15)	0.180 (64)	81.0%	0.009
		G		0.909 (149)	0.820 (292)	66.2%	
0301 02			A	0.683 (112)	0.618 (220)	66.3%	n.s.
			G	0.317 (52)	0.382 (136)	72.3%	
0301 02	total			(164)	(356)	68.5%	

Table 4

Proportion of DR3 B18 and DR3 B8 transmitted to T1D affected offspring carrying allele TNF and LTA SNPs. The frequency of not transmitted and transmitted haplotypes among specific DR-DQ-HLA-B haplotypes (counts in parentheses) are shown

DRB1 DQB1 B	LTA A1069G	TNF G (-238)A	TNF G (-308)A	Not Transmitted	Transmitted	% trans	P<
0301 02 B18	A			0.875 (14)	0.941 (64)	82.1%	n.s.
	G			0.125 (2)	0.059 (4)	66.7%	
0301 02 B18		A		0.875 (14)	0.882 (60)	81.1%	n.s.
		G		0.125 (2)	0.118 (8)	80.0%	
0301 02 B18			A	0.125 (2)	0.029 (2)	50.0%	0.11
			G	0.875 (14)	0.971 (66)	82.5%	
0301 02 B18	total			(16)	(68)	81.0%	
0301 02 B8	A			0.096 (11)	0.042 (9)	45.0%	0.050
	G			0.904 (104)	0.958 (207)	66.6%	
0301 02 B8		A		0.000 (0)	0.000 (0)	N/A	N/A
		G		1.000 (115)	1.000 (216)	65.3%	
0301 02 B8			A	0.887 (102)	0.931 (201)	66.3%	0.17
			G	0.113 (13)	0.069 (15)	53.6%	
0301 02 B8	total			(115)	(216)	65.3%	