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Family-based analysis of tumor necrosis factor and lymphotoxin-α tag polymorphisms with type 1 diabetes in the population of South Croatia

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Abstract

Tumor necrosis factor (TNF) and lymphotoxin-α (LTA) are cytokines with a wide range of inflammatory and immunomodulatory activities. Type 1 diabetes is an autoimmune disease characterized by destruction of insulin-producing pancreatic β cells. The aim of the present study was to evaluate the association of polymorphisms in the *TNF/LTA* gene region with susceptibility to type 1 diabetes. We investigated 11 *TNF/LTA* tag polymorphisms, designed to capture the majority of common variation in the region, in 160 trio families from South Croatia. We observed overtransmission of alleles from parents to affected child at five variants: (rs909253, allele C, $p = 1.2\times10^{-4}$; rs1041981, allele A, $p = 1.1\times10^{-4}$; rs1800629 (G-308A), allele A, $p = 1.2\times10^{-4}$; rs361525(G-238A), allele G, $p = 8.2\times10^{-3}$ and rs3093668, allele G, $p = 0.014$). We also identified overtransmission of the rs 1800629(G-308A)-rs361525(G-238A) A-G haplotype, $p = 2.384\times10^{-5}$. The present study found an association of the *TNF/LTA* gene region with type 1 diabetes. A careful assessment of *TNF/LTA* variants adjusted for linkage disequilibrium with *HLA* loci is needed to further clarify the role of these genes in type 1 diabetes susceptibility in the population of South Croatia.

Keywords

Type 1 diabetes; Tumor necrosis factor; Lymphotoxin alpha; TDT; Tag polymorphism

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.humimm.2008.12.010.

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1. Introduction

Type 1 diabetes mellitus (T1DM) results from a cellular-mediated autoimmune destruction of pancreatic β-cells [1]. It has a strong genetic component. The most important genetic factors for determining the risk of developing T1DM reside in the *HLA* class II loci but also, according to recent findings, in the *HLA-B* and *HLA-A* class I genes [2]. Several other gene regions have also been identified: a region 5′ to the *INS* gene, *CTLA4, PTPN22, IL2RA,* and the *IFIH1* region [3,4]. A recent T1DM genome-wide association study identified another four regions at 12q24, 12q13, 16p13, and 18p11 [5].

The region between *HLA* class I and II, known as class III, contains a number of genes involved in inflammatory and immune response such as tumor necrosis factor *(TNF)* and lymphotoxin-α (*LTA*) [6]. Both genes mediate similar pleiotropic effects and both bind the same TNF receptors [7]. Several activated immune system cells (monocytes, macrophages, and natural killer cells) release TNF, which then induces the expression of HLA class II molecules on the surface of the immune system cells [8,9]. TNF is believed to be one of the main proinflammatory cytokines implicated in the destruction of pancreatic β-cells [10]. However, TNF has opposing effects on T1DM. It is shown in an animal model that TNF can be, supported by both interleukin-1 and interferon-γ, toxic for β-cells [11]. Also, nonobese diabetic (NOD) mice that overexpressed TNF-α in their β-cells are predisposed to diabetes [12]. Neonatal TNF treatment of NOD mice results in earlier onset and increased incidence of T1DM, however, TNF administration to adult NOD mice prevents them from developing T1DM [10]. Also, administration of recombinant TNF to fetal thymus organ culture has a significant impact on the development of T cells such that they no longer cause T1DM [13]. The LTA cytokine is produced by lymphocytes and mediates a large number of inflammatory, immunostimulatory and antiviral responses [6]. The role of LTA was demonstrated as essential for the normal development of peripheral lymphoid organs in mice models [14]. It was shown that administration of LTA in NOD mice and Bio-Breeding (BB) rats may have modulated autoimmunity and prevented development of T1DM [15]. Feugeas *et al.* observed lower LTA production in T1DM patients than in normal controls, implying that a defect of LTA production can be a T1DM risk factor [7].

A genetic correlation between *TNF* and *LTA* genes with T1DM is widely suggested, although some reports show that the association between alleles at the *TNF/LTA* locus and T1DM can be attributed to linkage disequilibrium (LD) with the susceptible DQB1-DRB1 haplotypes, instead of an independent effect [6,16]. However, several other studies found an independent effect of *TNF/LTA* with T1DM [17-20]. TNF involvement in the destruction of pancreatic β-cells [10,11] and multiple inflammatory activities of TNF and LTA cytokines make them plausible targets for revealing molecular susceptibility to T1DM. The joint analysis of *TNF* and *LTA* genes may be more informative in defining the risk/protection to T1DM susceptibility [7,18].

The *TNF* and *LTA* genes lie next to each other on chromosome 6p21.3. According to Makhatadze there is a localization of *TNF* and *LTA* genes within the *HLA* complex, as shown in Figure 1 [8]. Two *TNF* gene promoter single nucleotide polymorphisms (SNPs), G-238A (rs361525) and G-308A (rs1800629), have frequently been studied [6,16-22]. However, inconsistencies in SNP designation through various *LTA* gene studies makes difficult to distinguish and differentiate investigated SNPs [6,7,18,22]. Studies in North Indian, Hungarian, Bahraini, and Moroccan populations observed associations of *TNF* -308 SNP and *TNF/LTA* haplotypes with T1DM independently of *HLA* loci [17-20,22]. On the other hand, *TNF/LTA* T1DM associations in Chinese, Caucasian, and Polish populations were explained by dependence on *HLA* alleles [6,16,21].

In this study we investigate 11 SNPs selected as tagging SNPs (tagSNP) for the *TNF* and *LTA* genes. These include the two extensively studied *TNF* promo ter SNPs (rs361525 and rs1800629), five other *TNF* SNPs (rs1800750, rs3093662, rs3093664, rs3093665, rs3093668), and four *LTA* SNPs (rs928815, rs909253, rs746868, rs1041981). These SNPs were selected to capture the majority of common variations in the *TNF/LTA* gene region.

The aim of the present study was to analyze the transmission of 11 *TNF/LTA* gene tag SNPs in case-parent trio samples and to evaluate their association with susceptibility to T1DM in the population of South Croatia.

2. Subjects and methods

2.1. Subjects

We typed 132 parent– offspring trios, 20 parent– offspring duos, and seven families with two and one family with three affected children from the population of South Croatia. Each proband was ascertained with T1DM according to the World Health Organization criteria. The sex distribution among affected children was 83 (49.11%) males and 86 (50.08%) females and the mean age at the onset of T1DM was 8.86 ± 5.36 (mean ± SD). Table 1 shows clinical, immunologic, and lifestyle characteristics of 88 of the patients who were involved in this study. Immunologic characteristics refer to all other immunologic diseases of patients beside T1DM, mostly Hashimoto thyreoiditis, asthma, and psoriasis. Clinical characteristics refer to history of other diseases of patients such as chicken pox, tonsillitis, scarlet fever, and various allergic reactions. We also show exposure to early introduction of milk formulas before 3 months of age and exposure to stress before the onset of diabetes such as the war in Croatia in the beginning of the 1990s, the loss of a dear person, and various family problems as lifestyle characteristics. We also show the area of living (urban/rural) of the patients. This study was approved by the ethics committee, and informed consent from patients and their parents was obtained prior to the blood sampling.

2.2. SNP selection and genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Eleven haplotype-tagging SNPs (rs928815, rs909253, rs746868, rs1041981, rs1800750, rs1800629, rs361525, rs3093662, rs3093664, rs3093665, rs3093668) were selected across the *TNF* and *LTA* loci from SNP data generated in 32 Caucasian mother–father–child trios as previously described [23,24]. Genotypes were determined using a fluorescence-based competitive allele specific assay (Kaspar, Kbioscience, UK). Using the same methodology, and as a measure of quality control, all samples were checked for gender. This assay investigates the single base difference between a homologous exon of the zinc-finger genes ZFX and ZFY and is diagnostic for sex chromosome (assay and SNP selection details are available from the authors on request). Gender control genotyping identified six discrepancies when compared to database information. These six individuals and their families were excluded from further analysis.

2.3. Statistical analysis

Before association analysis, we performed quality control of the obtained trio genotypes. We tested for Mendelian inheritance and identified inconsistencies in two families, which were then excluded from the analysis. We evaluated the rate of missing genotypes using Plink version 1.00 (http://pngu.mgh.harvard.edu/purcell/plink/) [25]. The percentage of missing genotypes for SNPs rs746868 and rs3093664 was 5.6% and 2.3%, respectively, but were included in further analyses. For the remaining nine SNPs the percentage of missing genotypes remained less than 1%. Hardy-Weinberg equilibrium (HWE) in healthy parents

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was tested using Pedstats [26]. Two SNPs showed a slight deviation from HWE in healthy parents (rs928815, $p = 0.039$ and rs746868, $p = 0.035$) but were included in further analyses. Minor allele frequencies (MAF) were compared with the National Center for Biotechnology Information SNP database (NCBI dbSNP) MAF for the central European (CEU) population (www.ncbi.nlm.nih.gov/projects/SNP/). MAF in healthy parents was not concordant with NCBI dbSNP frequencies for the CEU population for rs3093664 SNP (MAF_healhy_parents = 0.503, MAF_NCBI = 0.065). Because of the two quality control discrepancies (significant MAF difference with CEU population and a small but noticeable genotype missingness), rs3093664 SNP was excluded from the association analysis.

The proportion of variation across the *TNF/LTA* region captured by tagSNPs from this study was calculated based on the HapMap [27], using Tagger (http://www.broad.mit.edu/mpg/tagger/server.html) [28]. After quality control, a total of 152 cleaned-up families and 10 tagSNPs were taken forward to further association analyses.

Single-point and multi-point association analyses were carried out using implementations of the transmission disequilibrium test (TDT) in Plink and Unphased [25,29]. Haplotype frequencies were estimated using the expectation-maximization algorithm implemented in Unphased [29]. The r^2 and D′ measures of pairwise LD were calculated for all SNPs using Haploview [30]. Power calculations were performed using Quanto [31]. To obtain empirical p values, 10,000 permutations were run for each analysis, and permutation p values less than 0.05 were considered statistically significant.

To test whether our results would replicate in the largest genome-wide association analysis of T1DM, we applied for and received approval for Wellcome Trust Case Control Consortium (WTCCC) genotype data access [32]. We then conducted a case-control analysis in the WTCCC dataset of the same chromosomal region, as was investigated in our study, and compared the results.

3. Results

SNPs rs928815-rs746868, rs909253-rs1041981, and rs361525-rs3093668 were in tight LD with each other (D′ = 1, r^2 =1;D′ = 1, $r^2 = 0.984$ and D′ = 1, $r^2 = 0.797$, respectively). Other SNPs showed some or no LD between each other. LD correlation between 10 investigated *TNF/LTA* SNPs in healthy parents can be seen in Supplement 1, Figures 1a and 1b. Seven of our tag SNPs were found in the HapMap (build 35) database (rs928815, rs909253, rs1041981, rs1800750, rs1800629, rs3093662, rs3093668). Based on the HapMap data, these seven SNPs capture 21 polymorphic sites (>61.9% of common variation) in the region (chr6:31634835-31655771) at an r^2 threshold of greater than 0.8. The list of 21 *TNF/LTA* common variants and their 7 tagSNPs are shown in Supplement 2, Table 1.

The results of TDT single-point analysis, information about tagSNPs gene positions, allele changes and MAFs are shown in Table 2. We detected three statistically significant minor allele overtransmissions from parents to affected child for SNPs rs909253 ($p = 1.2\times10^{-4}$), rs1041981 ($p = 1.1\times10^{-4}$), and rs1800629 (G-308A) ($p = 1.2\times10^{-4}$) and two minor allele undertransmissions for SNPs rs361525 (G-238A) ($p = 8.2\times10^{-3}$) and rs3093668 ($p = 0.014$) after 10,000 permutations. Genotype data for affected children and their parents can be seen in Supplement 3, Table 2.

Haplotype-based TDT analysis showed T1DM association with rs1800629(G-308A) and rs361525(G-238A) haplotypes ($p = 2.384\times10^{-5}$), after 10,000 permutations. Specifically, the A-G rs1800629(G-308A)-rs361525(G-238A) haplotype was overtransmitted from parents to affected offspring (Table 3).

None of our 10 investigated SNPs were analyzed in the WTCCC study of T1DM. This is because WTCCC genotyping platform, Affymetrix 500 K, did not have these SNPs incorporated within. There was only one SNP from *TNF* gene (rs1799964) that was part of Affymetrix 500 K chip. SNP rs1799964 showed significant association with T1DM in WTCCC case-control analysis (OR = 1.303, $p = 3.948\times10^{-8}$). However, based on the HapMap database rs1799964 is not in LD with any of our 10 *TNF/LTA* SNPs (Supplement 4, Figure 2), therefore we could not make any comparison of our association results with WTCCC results.

4. Discussion

In this study we analyzed 10 *TNF/LTA* gene SNPs with T1DM and found significant association in the families from South Croatia. SNPs from this study were designed to tag most of the common variation from the *TNF/LTA* gene region and were analyzed in T1DM for the first time. We detected three minor (rs909253, rs1041981, rs1800629 (G-308A)) and two major (rs361525 (G-238A), rs3093668) allele overtransmissions from parents to affected children. We also observed significant A-G rs1800629(G-308A)-rs361525(G-238A) haplotype overtransmission. This study had 90% statistical power to detect an effect (at $\alpha = 0.05$) for SNPs rs909253, rs1041981, and rs1800629 and less than 80% power for SNPs rs361525(G-238A) and rs3093668 assuming an additive model.

Two SNPs that show overtransmission of the minor allele (rs909253, rs1041981) and two that overtransmit a major allele (rs361525(G-238A), rs3093668) are in LD with each other (Supplement 1). It is possible that only one SNP drives the association and the observed patterns are, consequently, caused by LD. Other SNP that shows very significant minor allele overtransmission, rs1800629(G-308A) is not in LD with any other investigated SNP. Interestingly, overtransmission of A-G rs1800629(G-308A)-rs361525(G-238A) haplotype shows stronger significance (2.384×10^{-5}) than any of the single markers individually (rs1800629 (G-308A), $p = 1.2\times10^{4}$; rs361525(G-238A), $p = 8.15\times0^{-3}$). As both promoter polymorphisms have been associated with transcriptional enhancement rate it is possible that when acting *in cis* these two markers show an even stronger interaction [33-35].

The *HLA* class III region, particularly around *TNF*, has been regarded as a susceptibility locus for T1DM [36]. The most frequently analyzed SNPs from this region are the two *TNF* promoter SNPs at positions −308 (rs1800629) and −238 (rs361525). *LTA* lies next to *TNF*, and several studies have combined *TNF/LTA* SNPs and analyzed them with respect to T1DM. A significant increase in *TNF* −308 allele A and G/A and A/A genotypes was found in North Indian T1DM cases [17,19]. Similarly, an increase in the prevalence of the *TNF* −308 A allele was reported in Hungarian diabetic patients [20]. Associations of the *TNF/LTA* haplotypes and *LTA* +249A/G SNP with T1DM, was suggested to be independent of *HLA* in the Moroccan and Bahraini populations [18,22]. On the other hand, Deng *et al.* excluded the −308 polymorphism as a marker forT1DM in Chinese and Caucasian datasets because of LD with the DR3-DQB1*0201 haplotype [21]. In addition, after adjusting the data for LD with DRB1-DQB1 and B18-DR3 haplotypes, Noble *etal.* observed a lack of *TNF* −308, −238, and *LTA* A1069G SNP association with T1DM [6]. The similar conclusions were drawn out from the *TNF* studies in southwestern Polish and in Japanese populations [16,33].

Several studies reported a small independent effect of *TNF/LTA* SNPs, particularly of rs1800629 (−308), on susceptibility to T1DM. In our study we also observed associations of several *TNF/LTA* SNPs, including of rs1800629 (−308), with T1DM. However, the South Croatian families analyzed in this study have not been *HLA* typed and therefore we can not determine whether the effects of the *TNF/LTA* variants are primary or secondary to *HLA*

loci. A functional support for independent effects may be explained by transcription rate enhancement of *TNF* gene by *TNF* promoter −308 and −238 SNPs, functional control of *TNF* promoter SNPs on *LTA* expression and expression of other nearby genes [34,35,37-39]. An integrated role of TNF and other inflammatory cytokines in the destruction of pancreatic beta cells [19] provides further argument for a *TNF/LTA* T1DM effect.

In summary, the present study investigated the association of 10 *TNF/LTA* tag SNPs, specifically designed to capture the majority of common variation in the region, with T1DM in families from South Croatia. Selected SNPs were analyzed with T1DM for the first time. We observed associations of variants in the *TNF/LTA* gene region with T1DM. However, to fully understand the *TNF/LTA* involvement in T1DM susceptibility, a careful assessment of *TNF/LTA* genes adjusted for LD with *HLA* in a larger sample is needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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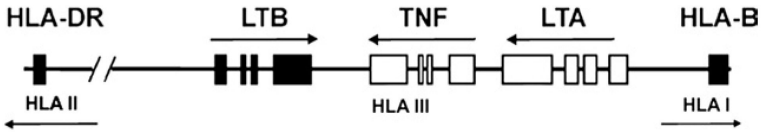

Fig. 1.
Localization of *TNF* and *LTA* genes inside *HLA* complex, according to Makhatadze [8].

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Table 1

Clinical, immunological and some life style characteristics of 88 patients who were involved in the study of TNF/LTA genes

Characteristic	No. of patients (%) *N* = 88
Positive T1DM family history	40 (45.4)
Other autoimmune diseases	14 (15.9)
Introduction of milk formulas before 3 months of age	50 (56.8)
Chicken pox	34 (48.9)
Tonsillitis	29 (32.9)
Scarlet fewer	5 (5.7)
Allergic reactions	13 (14.7)
Stress before T1DM onset	33 (37.5)
Urban area of living	55 (62.5)

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Table 2

Transmission disequilibrium analysis of 10 TNF/LTA SNPs in 152 parent-offspring trio families

SNP	Position[a]	MAF[b]	M:C[c]	T:U[d]	OR[e]	L95[e]	U95[e]	χ^2 [f]	*p* Value[g]
rs928815	5′ *LTA*	0.406	T:G	48:69	0.6957	0.4813	1.005	3.769	0.052
rs909253	*LTA*-intron 1	0.396	C:T	81:39	2.077	1.417	3.043	14.7	0.00012
rs746868	*LTA*-intron 1	0.400	C:G	40:59	0.678	0.4538	1.013	3.646	0.056
rs1041981	*LTA*-exon 3	0.391	A:C	80:38	2.105	1.431	3.097	14.95	0.00011
rs1800750	5′ of *TNF*	0.003	A:G	0:1	/	/	/	1	0.317
rs1800629	5′ of *TNF*	0.269	A:G	65:28	2.321	1.491	3.616	14.72	0.00012
rs361525	5′ of *TNF*	0.006	A:G	0:7	/	/	/	7	0.00815
rs3093662	*TNF*-intron 1	0.038	G:A	9:16	0.5625	0.2486	1.273	1.96	0.162
rs3093665	*TNF*-3′UTR	0.032	C:A	9:9	1	0.397	2.519	0	1
rs3093668	3′ of *TNF*	0.003	C:G	0:6	/	/	/	6	0.014

[a] Position of the SNP within the gene.

[b] MAF in T1DM children.

[c] Minor allele (M) vs. common allele (C).

[d] Copies of the minor allele transmitted (T) and nontransmitted (U).

[e] Odds ratios (OR) with 95% lower and upper confidence intervals (L95,U95).

[f] Chi square (χ^2) test.

[g] *p* Value after 10,000 permutations.

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Table 3

Transmission of rs1800629(G-308A)-rs361525(G-238A) haplotypes in152 parent–offspring trio families

Haplotype	T	Freq T	NT	Freq NT	OR	*p* Value[a]
1-2 (A-G)	72	0.2571	35	0.125	1	2.384×10^{-5}
2-1 (G-A)	1	0.0035	8	0.028	0.6	
2-2 (G-G)	207	0.7393	237	0.8464	0.4	

T, transmitted; freq T, frequency transmitted; NT, nontransmitted; freq NT, frequency nontransmitted; OR, odds ratio.

[a]overall p-value.