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*Fok*I Polymorphism, Vitamin D Receptor, and Interleukin-1 Receptor Haplotypes Are Associated with Type 1 Diabetes in the Dalmatian Population

Tatijana Zemunik,* Veselin Škrabić,† Vesna Boraska,* Dijaneta Diklić,* Ivana Marinović Terzić,* Vesna Čapkun,‡ Marijana Peruzović,* and Janoš Terzić*

From the Departments of Biology and Physiology, School of Medicine, University of Split, Split; and the Departments of Pediatrics† and Nuclear Medicine,‡ Clinical Hospital in Split, Split, Croatia*

Vitamin D and interleukin (IL)-1 have been suggested to function in the pathogenesis of type 1 diabetes mellitus (T1DM). Therefore, we examined the influence of gene polymorphisms in vitamin D receptor (VDR) and interleukin-1 receptor type I (IL-1-R1) on susceptibility to T1DM in the Dalmatian population of South Croatia. We genotyped 134 children with T1DM and 132 controls; for *Fok*I polymorphism studies, we extended the control group to an additional 102 patients. The VDR gene polymorphism *Fok*I displayed unequal distribution ($P = 0.0049$) between T1DM and control groups, with the ff genotype occurring more frequently in T1DM individuals whereas the VDR gene polymorphism *Tru*9I did not differ in frequency between studied groups. All tested polymorphisms of the IL-1-R1 gene [*Pst*I, *Hin*fI, and *Alu*I (promoter region) and *Pst*I-e (exon 1B region)] displayed no differences between cases and controls. Haplotype analysis of the VDR gene (*Fok*I, *Bsm*I, *Apa*I, *Taq*I, *Tru*9I) and of the IL-1-R1 gene (*Pst*I, *Hin*fI, *Alu*I, *Pst*I-e) found haplotypes VDR FbATu ($P = 0.0388$) and IL-1-R1 phap' ($P = 0.0419$) to be more frequent in T1DM patients whereas the BatU haplotype occurred more often in controls ($P = 0.0064$). Our findings indicate that the VDR *Fok*I polymorphism and several VDR and IL-1-R1 haplotypes are associated with susceptibility to T1DM in the Dalmatian population. *(J Mol Diagn 2005, 7:600–604)*

Type 1, or insulin-dependent, diabetes mellitus (T1DM) presents with ketosis-prone hyperglycemia associated with an almost complete loss of insulin-producing pancreatic β cells. T1DM is a multifactorial autoimmune disease for which susceptibility is determined by both environmental and genetic factors.[1–4] There are differences in the prevalence of T1DM in different populations, with disease being more common in Europeans and less prevalent in Asians and Africans.[5]

The main genetic contribution to T1DM susceptibility resides in the major histocompatibility complex.[6,7] Also, several non-major histocompatibility complex regions are involved, particularly the vitamin D receptor (VDR) gene and the interleukin-1 receptor type 1 (IL-1-R1) gene.[3,8–11] Vitamin D exerts its genomic action via the nuclear VDR. Several epidemiological and physiological studies support possible involvement of vitamin D in the development of T1DM. Vitamin D has immunosuppressive effects, inhibiting lymphocyte activation and cytokine and immunoglobulin production.[12] Infants who received vitamin D have a lower incidence of T1DM in adulthood.[13] Furthermore, administration of vitamin D to pregnant women lowers the incidence of T1DM in their offspring.[14]

The VDR gene is located on chromosome 12q (12-12q14) and is highly polymorphic.[15] There are six VDR polymorphisms that have been mainly studied: the *Fok*I restriction fragment length polymorphism (RFLP) in exon 2; *Bsm*I, *Tru*9I, and *Apa*I RFLPs located between exons 8 and 9; the *Taq*I restriction site in exon 9; and the poly-A polymorphism downstream of the 3′ untranslated region.[16] However, the four polymorphisms located at the 3′ end of the VDR gene [*Bsm*I (A to G), *Tru*9I (G to A), *Apa*I (G to T), and *Taq*I (T to C)] have unknown functional effects.[17] In addition, the VDR gene contains two potential translation initiation (ATG) sites.[18] The *Fok*I polymorphism, which occurs at the first start codon in exon 2, changes the nucleotide sequence to ACG.[19] Alleles with this polymorphism initiate translation three codons downstream, resulting in a protein (424 amino acids, the F allele) that is three amino acids shorter than wild-type and is considered to be more active.[17,20] This is the only known protein polymorphism in the VDR gene.[17,21] Association between some VDR gene polymorphisms and T1DM have been investigated and found to influence susceptibility to T1DM in several populations.[15,16,22–24]

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T.Z., V.S., and V.B. contributed equally to the study.

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Address reprint requests to Janoš Terzić, M.D., Ph.D., University of Split, School of Medicine, Department of Physiology, Šoltanska 2, 21000 Split, Croatia. E-mail: jterzic@bsb.mefst.hr.

IL-1 plays a central role in immune and inflammatory responses and has been proposed to be a mediator in a number of inflammatory diseases including T1DM.[25] Two IL-1 receptors, type 1 and 2, are encoded by two separate genes.[26] The type 1 receptor is found on most cells, including T lymphocytes and β cells, whereas the type 2 receptor is found primarily on neutrophils, monocytes, bone marrow cells, and B lymphocytes.[27] IL-1 in very low concentrations has cytotoxic effects on islets of Langerhans.[28] Also, compared to other cell types, pancreatic islet β cells are very sensitive to IL-1-induced changes in their functional activity.[29] The receptor IL-1-R1 gene maps to chromosome 2 (2q12-22) and is a large, complex gene, spanning ~74 kb and comprising 12 exons and three distinct promoters.[30–33] There are conflicting reports as to whether the IL-1-R1 gene is implicated in genetic susceptibility to T1DM.[10,34,35] Bergholdt and colleagues[34,35] have reported an association of *Pst*I and *Hin*fI polymorphisms in the IL-1-R1 promotor region to T1DM in a Danish population. Promoter IL-1-R1 gene polymorphisms *Hin*fI (G to A), *Pst*I (G to A), and *Alu*I (T to C) are not known to affect transcriptional activity; thus, there is no evidence to support any functional significance of the identified polymorphisms.[35] The *Pst*I-e polymorphism in exon 1B has been associated with T1DM in a British data set but not in the high-risk Finnish or low-risk South Indian population.[10] In the high-risk population of Finland, this polymorphism only associated with T1DM in patients lacking high-risk HLA-DR3 and DR4 haplotypes.[10] The IL-1-R1 *Pst*I-e polymorphism, which is situated in exon 1B of the gene, is caused by a C to T transition that does not affect any of the recognition sites for known transcriptional regulatory factor. Also, the different allelic variants do not appear to affect the predicted mRNA secondary structure.[10]

As we have previously studied *Bsm*I, *Apa*I, and *Taq*I VDR polymorphisms and their influence on T1DM susceptibility in the Dalmatian population, we extended our studies to the genotyping of the *Fok*I and *Tru*9I polymorphisms in the same study population. We also analyzed *Pst*I, *Hin*fI, and *Alu*I polymorphisms in the promoter region of IL-1-R1 gene and the *Pst*I-e polymorphism in the exon 1B region of the same gene. The aim of the present study was also to evaluate the strength of the linkage disequilibrium (LD), ie, allelic association between investigated polymorphisms, and to correlate LD with the disease. We also performed haplotype analysis of the VDR gene (by inclusion of the results for *Bsm*I, *Apa*I, *Taq*I) and of the IL-1-R1 gene and examined their influence on susceptibility to T1DM.

Materials and Methods

From the Dalmatian region of South Croatia, 134 unrelated patients (72 boys and 62 girls) were recruited for the study. T1DM was diagnosed according to the World Health Organization criteria.[12] The mean age at onset of T1DM was 8.6 ± 4.3 years (mean ± SD). Informed consent from patients and their parents was obtained before blood sampling. The control group, 132 unrelated, consecutive normal patients (62 boys and 70 girls), were recruited from individuals who visited the hospital for general health check-ups (mean age, 8.2 ± 4.9 years). For the analysis of the *Fok*I polymorphism, we extended the control group with 102 healthy blood donors from the Dalmatian region.

Genomic DNA was extracted from peripheral blood leukocytes using the Perfect gDNA kit (Eppendorf, Hamburg, Germany). Genotypes for four gene polymorphisms in IL-1-R1 gene (*Pst*I, *Hin*fI, *Alu*I, and *Pst*I-e) and two gene polymorphisms in VDR gene (*Fok*I and *Tru*9I) were identified by polymerase chain reaction followed by RFLP analysis, according to previous reports.[16,35] Genotypes were designated with a capital letter for the absence of a restriction site and with a lowercase letter for its presence. Therefore, the genotypes are as follow: *Fok*I = FF, ff (homozygous for the absence or presence of the cut site, respectively), and Ff (heterozygous); *Tru*9I = UU, uu, Uu; *Pst*I = PP, pp, Pp; *Hin*fI = HH, hh, Hh; *Alu*I = AA, aa, Aa; *Pst*I-e = P'P', p'p', P'p'. The digested fragments were separated in 3% agarose gels and 8% acrylamide gels and were visualized by ethidium bromide staining. The EH+ program was used to estimate haplotype frequencies and the disequilibrium measure for VDR and IL-1-R1 gene polymorphisms.[36] Conclusions about association of certain haplotypes with susceptibility to T1DM have been derived from the χ^2 test. A comparison between groups was made on the basis of χ^2 test and power calculation. We used the Statistica 6.0 (StatSoft, Inc., Tulsa, OK) software package for statistical analysis. A P value of less than 0.05 was considered statistically significant.

Results

The frequency of the VDR and IL-1-R1 gene polymorphisms in the T1DM patients and controls is shown in Table 1. The distribution of genotype frequencies of the *Fok*I VDR polymorphism among T1DM patients and controls differed significantly ($\chi^2 = 10.646$, $P = 0.0049$) with the ff genotype occurring more frequently in the T1DM patients. Another VDR gene polymorphism, *Tru*9I, did not differ in frequency between both studied groups. There were no differences in the genotype frequencies of the *Pst*I, *Hin*fI, and *Alu*I polymorphisms of promoter region or the *Pst*I-e polymorphism of exon 1B region of IL-1-R1 gene in T1DM patients and controls (Table 1). As genotype frequencies for the *Alu*I polymorphism were at the limit of statistical significance, $P = 0.07$, we performed power calculation and found no association between this polymorphism and T1DM (power = 0.606).

Recently, we analyzed several other VDR gene polymorphisms (*Apa*I, *Bsm*I, and *Taq*I) among the same study participants.[24] Thus, we were able to combine results from that study with our new results, enabling estimation of haplotype frequencies present among tested individuals. *Bsm*I, *Apa*I, *Taq*I, and *Tru*9I polymorphisms were found to possess a strong LD ($P < 0.0001$) whereas no significant LD between *Fok*I and any other polymorphism was detectable. The most frequent haplotypes in our population were BAtU (35%) and baTU (29%). The BatU

Table 1. Vitamin D Receptor and Interleukin 1 Receptor Type 1 Genotype Frequency in Dalmatian Population among T1DM Patients and Controls

Restriction site and genotype		T1DM, no. (%) $n = 134$	Controls, no. (%) $n = 232$	χ^2	P value (df = 2)
VDR*					
*Fok*I	ff	29 (22)	23 (10)		
	FF	42 (31)	73 (31)	10.64	0.0049
	Ff	63 (47)	136 (59)		
*Tru*9I	uu	3 (2)	4 (3)		
	UU	105 (78)	90 (68)	3.53	0.17
	Uu	26 (20)	38 (29)		
IL-1-R1†					
Promoter region					
*Pst*I	pp	73 (54)	72 (55)		
	PP	9 (7)	7 (5)	0.25	0.88
	Pp	52 (39)	53 (40)		
*Hin*fI	hh	75 (56)	76 (57)		
	HH	11 (8)	9 (7)	0.2	0.9
	Hh	48 (36)	47 (36)		
*Alu*I	aa	28 (21)	34 (26)		
	AA	20 (15)	31 (23)	5.29	0.07
	Aa	86 (64)	67 (51)		
Exon 1B region					
*Pst*I-e	p'p'	51 (38)	54 (41)		
	P'P'	13 (10)	16 (12)	0.87	0.65
	P'p'	70 (52)	62 (47)		

*VDR–vitamin D receptor
†IL-1-R1–interleukin-1 type I receptor

haplotype was observed in 10 controls but in no T1DM individuals ($P = 0.0064$). When integrated with the *Fok*I polymorphism, several other allele combinations were also found to be more common in the control group, such as FbATu ($P = 0.00163$) and fBATU ($P = 0.0374$). On the other hand, the FbATU ($P = 0.0388$) haplotype was found to be a risk factor for susceptibility to T1DM.

The IL-1-R1 gene polymorphisms *Pst*I, *Hin*fI, *Alu*I, and *Pst*I-e were also found to possess a strong LD ($P < 0.0001$). The most frequent haplotype in our population was PHap' (39%) followed by PHAP' (21%) and phAp' (20%). The allelic combination phap' was found to be linked with T1DM ($P = 0.0419$). Genotype frequencies for each RFLP fit the Hardy-Weinberg equilibrium except for the *Fok*I polymorphism in which we found a slight deviation among controls. For this reason, we extended the control group to an additional 102 new patients, but the deviation remained.

Discussion

We have found an association between the VDR *Fok*I polymorphism and T1DM in our study population ($\chi^2 = 10.646$, $P = 0.0049$). The genotype ff was twice as common in T1DM patients as in controls. Because the f allele corresponds to a less active VDR protein,[17,37] the ff genotype might contribute to the development of the T1DM either by causing weaker insulin production or by affecting vitamin D immunosuppressive properties.[38] The extended control group for the VDR *Fok*I polymorphism did not fit into Hardy-Weinberg equilibrium, and we explain this by hidden subpopulation stratification.

Several other studies found the *Fok*I polymorphism to contribute to genetic heterogeneity of T1DM. In agreement with our results, the less active VDR protein occurs more frequently in T1DM patients and also associates with GAD65-Ab-positive T1DM in the Japanese population,[16] although it was not informative in the German population.[15] However, a similar study was performed in two Spanish populations with different genetic backgrounds: the Mediterranean population (Barcelona) exhibited no difference regarding *Fok*I polymorphism whereas the Navarra population exhibited decreased ff genotype (the less active form) in T1DM patients.[39]

We were also able to compute the strength of LD between studied polymorphisms and to estimate the haplotype frequencies. The observed LD between *Bsm*I, *Apa*I, *Taq*I, and *Fok*I are in accordance with previously reported findings for Caucasian populations.[27,40–43] The most frequent haplotypes were BAtU (35%) and baTU (29%), which is also a confirmation of the previous findings for Caucasians.[24,40] *Bsm*I, *Apa*I, *Taq*I, and *Tru*9I RFLPs are located near the 3′ end of the VDR gene, which contains many polymorphisms, and the LD extends into the 3′ regulatory region containing the UTR. The 3′ UTR of genes is known to be involved in regulation of expression, especially through regulation of mRNA stability and degradation.[17] Although the RFLPs we studied were anonymous, ie, have no known functional effect, the associations we found might be explained by their strong LD with some truly functional polymorphism in the 3′ UTR region.

The VDR protein can exist in less active (f allele) and more active (F allele) variants. Individuals with the identical heterozygous genotypes for *Fok*I, *Bsm*I, *Apa*I, and *Taq*I might produce different levels of VDR protein. This occurs as a result of the particular haplotype combinations, which produce more or less active VDR protein

depending on their linkage with the 3′ UTR.[17,44] This could not have been predicted by analyzing single SNPs and/or by looking at genotypes of individual SNPs but is only evident on analysis of gene-wide haplotypes.[17]

In light of these descriptions, we can offer an explanation for why two of our possibly protective haplotypes, FbATu and fBATU, carry different *Fok*I alleles. The fBATU haplotype carries the f allele, which is a susceptibility allele in our population; however, this haplotype is found more commonly in the control group. One possible explanation is that the BATU haplotype is linked with some truly functional polymorphism in the 3′ UTR region that determines higher transcription and/or stability of mRNA and diminishes the effect of susceptibility of the f allele and of a less active VDR protein.

This study also demonstrates that IL-1-R1 gene single polymorphisms are not associated with an increased risk of T1DM in the Dalmatian population. Thus far the *Hin*fI polymorphism of the promoter region has been linked with T1DM whereas *Pst*I and *Alu*I polymorphism have not.[30] In the high-risk population of Finland, the *Pst*I-e polymorphism in exon 1B of the gene only showed association to T1DM in patients without high-risk HLA-DR3 and DR4 haplotypes.[10]

However, the results change when looking at the haplotype level. We found strong LD between all four studied polymorphisms within the IL-1R1 gene. Cox and colleagues[6] reported a strong LD between *Pst*I and *Hin*fI polymorphisms but not between *Pst*I and *Alu*I. One specific allele combination, phap', is distinguished as being more frequent in T1DM patients of the Dalmatian population. This could be explained with the extended LD throughout the region; thus, the studied polymorphisms could be in LD either with a mutation in a regulatory region of the IL-1-R1 gene or with a different disease locus in this chromosomal region.[25] It has been suggested that the haplotypes, rather than individual genes, incorporating different allelic variants of genes in the IL-1 gene cluster may be important in determining susceptibility to T1DM.[25] To our knowledge this is the first report of linkage of haplotypes of these four IL-1-R1 gene polymorphisms with T1DM.

The results of our study provide more evidence of the polygenic nature of T1DM and of the genetic heterogeneity between different populations. We confirm that in the Dalmatian population of South Croatia the VDR *Fok*I polymorphism and several VDR and IL-1-R1 haplotypes correlate with the susceptibility to and development of T1DM.

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