Human Immunology (2008) 69, 134–138

journal homepage: www.elsevier.com/locate/humimm

Lack of association of vitamin D receptor gene polymorphisms with susceptibility to type 1 diabetes mellitus in the Portuguese population

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Received 15 November 2007; received in revised form 17 January 2008; accepted 23 January 2008

KEYWORDS
Vitamin D receptor;
VDR;
Single nucleotide polymorphism;
SNP;
Type 1 diabetes mellitus;
Genetic susceptibility

Summary The vitamin D receptor (*VDR*) gene is a candidate gene for susceptibility to autoimmune disorders. Association studies of *VDR* polymorphisms and risk of type 1 diabetes often produced conflicting results in different ethnic backgrounds. The aim of this study was to test for association between common *VDR* polymorphisms and the genetic susceptibility to type 1 diabetes in the Portuguese population. We genotyped 207 patients with type 1 diabetes and 249 controls for the *Fok*I T>C (rs10735810), *Bsm*I A>G (rs1544410), *Apa*I G>T (rs7975232), and *Taq*I C>T (rs731236) single nucleotide polymorphisms by polymerase chain reaction and restriction fragment length polymorphism analysis. The distribution of *VDR* genotype, allele, and haplotype frequencies did not differ significantly between patients and controls. These data suggest that the single nucleotide polymorphisms of the *VDR* gene are unlikely to contribute significantly to type 1 diabetes susceptibility in the Portuguese population.

Introduction

Vitamin D is a potent modulator of the immune system and is involved in the regulation of cell proliferation and differentiation [1]. Vitamin D is an effective immunosuppressant via inhibition of lymphocyte activation and cytokine production [1] and prevents or markedly suppresses the development of several autoimmune diseases in animal models [1]. The administration of vitamin D protects against the development of insulitis and type 1 diabetes in nonobese diabetic mice [2]. In humans, epidemiological studies indicated that dietary vitamin D supplementation during early childhood decreases the risk of type 1 diabe-

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doi:10.1016/j.humimm.2008.01.008

ABBREVIATIONS

BP	base pairs
PCR	polymerase chain reaction
SNP	single nucleotide polymorphism
VDR	vitamin D receptor

tes [3,4] and that maternal intake of vitamin D during pregnancy may have a protective effect on the appearance of islet autoantibodies in offspring [5].

Because vitamin D exerts its effects through the vitamin D receptor (VDR), the *VDR* gene has become a candidate susceptibility gene for type 1 diabetes. The *VDR* gene is located on chromosome 12q12–q14 and includes eight protein-coding exons (exons 2–9) and six untranslated exons (exons 1a–1f), which are alternatively spliced [6]. Four common single nucleotide polymorphisms (SNPs) in the *VDR* gene have been investigated extensively: *Fok*I T>C (rs10735810), *Bsm*I A>G (rs1544410), *Apa*I G>T (rs7975232), and *Taq*I C>T (rs731236). Allele T of the *Fok*I SNP creates an alternative ATG initiation codon in exon 2 leading to a VDR protein that is three amino acids longer [7]. The *Bsm*I and *Apa*I SNPs are both located in intron 8, and the *Taq*I is a silent SNP in exon 9. The four SNPs were tested for association with various human diseases [8] and affected the risk of endocrine immune-mediated disorders such as Graves' disease, Hashimoto's thyroiditis and Addison's disease [8–10]. Several studies also reported the association of type 1 diabetes with one or more of the four SNPs. However, the reported associations are inconsistent among studies [11].

The aim of this study was to assess the contribution of these *VDR* polymorphisms to the susceptibility to type 1 diabetes in the Portuguese population.

Subjects and methods

Subjects

The study group consisted of 207 Caucasian Portuguese patients with type 1 diabetes mellitus (113 males and 94 females; mean age at time of study ± SD = 27.5 ± 10.2 years) who attended the outpatient clinics at the University Hospital of Coimbra (Portugal). Diabetes was diagnosed according to World Health Organization criteria [12] and classified as type 1 on the basis of classical clinical presentation [12], low or undetectable levels of serum C-peptide, and the presence of one or more autoantibodies (islet cell autoantibodies, autoantibodies to insulin, autoantibodies to glutamic acid decarboxylase (GAD65), or autoantibodies to the tyrosine phosphatase IA-2). Mean duration of diabetes was 11.4 ± 8.3 years (mean ± SD). The control group consisted of 249 unrelated volunteers (143 males and 106 females; mean age ± SD = 36.8 ± 13.8 years) who were blood donors and hospital and faculty staff with no history of autoimmune or other chronic diseases from the same geographical region. Portugal has approximately 10 million inhabitants and the annual incidence rate for type 1 diabetes was estimated at about 10 per 100,000 [13]. Informed consent was obtained from patients and controls.

Genotyping

Genomic DNA was extracted from whole blood using standard protocols. DNA was amplified by polymerase chain reaction (PCR) using previously described primer sequences [14]. Amplified fragments were digested with the appropriate restriction enzyme (New England Biolabs, Beverly, MA, USA) according to the manufacturer's instructions and visualized on a 3% agarose gel. The *Fok*I T>C (rs10735810) SNP was analyzed by digestion of a 267-base pair (bp) PCR product with *Fok*I, which resulted in two fragments of 206 and 61 bp in the presence of the T allele and in an uncut fragment in the presence of the C allele. The *Bsm*I A>G (rs1544410) SNP was analyzed by digestion of a 191-bp PCR product with *Bsm*I, which resulted in two fragments of 115 and 76 bp in the presence of the G allele and in an uncut fragment in the presence of the A allele. The *Apa*I G>T (rs7975232) and *Taq*I C>T (rs731236) SNPs were analyzed by digestion of a 745-bp PCR product with *Apa*I, which resulted in two fragments of 528 and 217 bp in the presence of the G allele and in an uncut fragment in the presence of the T allele, and by digestion with *Taq*I, which resulted in three fragments of 293, 251, and 201 bp in the presence of the C allele and in two fragments of 494 and 251 bp in the presence of the T allele. *VDR* haplotypes derived from *Fok*I, *Bsm*I, *Apa*I, and *Taq*I polymorphisms were constructed using informative combinations of genotypes (e.g., an individual genotyped as CT/AA/GG/TT was considered to possess haplotypes C/A/G/T + T/A/G/T). Individuals heterozygous for more than one polymorphic site were not considered for haplotype frequency analysis, because the distribution of the alleles between the two homologous chromosomes could not be unequivocally defined (e.g., an individual genotyped as CT/AG/GG/TT could possess either haplotypes C/A/G/T + T/G/G/T or C/G/G/T + T/A/G/T).

Statistical analysis

Pearson's χ^2 test of independence, with one degree of freedom, was used to examine differences of genotype, allele, and haplotype frequencies between patients and controls. When expected values were less than 5, Fisher's exact test was used. Two-tailed p values were calculated and statistical significance was set at $p < 0.05$. Odds ratios and the corresponding 95% confidence intervals were calculated for each genotype, allele, and haplotype. Hardy-Weinberg equilibriums were assessed using the χ^2 goodness of fit test to compare the observed and allele-based expected genotype frequencies. Power calculation was analyzed using the program Power and Sample Size Calculations (Version 2.1.30).

Results

All frequencies were in Hardy–Weinberg equilibrium. The distribution of *VDR* genotype and allele frequencies did not differ significantly between patients with type 1 diabetes mellitus and controls (Table 1). No single genotype or allele was associated with an altered risk for type 1 diabetes mellitus. Because there were three possible genotypes for each of the four SNPs, each individual was classified as having one of the 81 possible combinations of genotypes. The distribution of these genotype combinations did not differ between patients and controls (data not shown). *VDR* haplotypes could be deduced from informative combinations of genotypes in 44.9 and 44.8% of patient and control haplotypes, respectively. *VDR* haplotype frequencies did not differ significantly between patients and controls (Table 2). Power analysis demonstrated that the study sample size was sufficient to detect odds ratios of 1.45 to 1.49 for each studied

Table 1 *VDR* genotype and allele frequencies in patients with type 1 diabetes mellitus and controls.

Polymorphism	Controls, *n* (%)	Patients, *n* (%)	Odds ratio (95% CI)	*p* value
*Fok*I genotype	*n* = 249	*n* = 207		
CC	97 (38.9)	81 (39.1)	1.01 (0.69-1.47)	0.970
CT	114 (45.8)	101 (48.8)	1.13 (0.78-1.63)	0.522
TT	38 (15.3)	25 (12.1)	0.76 (0.45-1.31)	0.327
*Fok*I allele				
C	308 (61.8)	263 (63.5)	1.07 (0.82-1.41)	0.602
T	190 (38.2)	151 (36.5)	0.93 (0.71-1.22)	0.602
*Bsm*I genotype	*n* = 248	*n* = 207		
AA	56 (22.6)	43 (20.8)	0.90 (0.58-1.41)	0.642
AG	107 (43.1)	96 (46.4)	1.14 (0.79-1.65)	0.490
GG	85 (34.3)	68 (32.8)	0.94 (0.64-1.39)	0.749
*Bsm*I allele				
A	219 (44.2)	182 (44.0)	0.99 (0.76-1.29)	0.954
G	277 (55.8)	232 (56.0)	1.01 (0.78-1.31)	0.954
*Apa*I genotype	*n* = 232	*n* = 205		
GG	63 (27.2)	50 (24.4)	0.87 (0.56-1.33)	0.510
GT	101 (43.5)	100 (48.8)	1.24 (0.85-1.80)	0.272
TT	68 (29.3)	55 (26.8)	0.88 (0.58-1.34)	0.565
*Apa*I allele				
G	227 (48.9)	200 (48.8)	0.99 (0.76-1.30)	0.967
T	237 (51.1)	210 (51.2)	1.01 (0.77-1.31)	0.967
*Taq*I genotype	*n* = 232	*n* = 205		
CC	46 (19.8)	41 (20.0)	1.01 (0.63-1.61)	0.964
CT	95 (41.0)	94 (45.9)	1.22 (0.84-1.78)	0.302
TT	91 (39.2)	70 (34.1)	0.80 (0.54-1.19)	0.272
*Taq*I allele				
C	187 (40.3)	176 (42.9)	1.11 (0.85-1.46)	0.432
T	277 (59.7)	234 (57.1)	0.90 (0.69-1.18)	0.432

n = number; CI = confidence interval.

allele, with an estimated power of 0.8 and a type 1 error probability of 0.05.

Discussion

The *VDR* locus has been studied in different populations for association with susceptibility to immune-mediated diseases, but with inconsistent findings in type 1 diabetes mellitus [11]. To clarify the contribution of *VDR* polymorphisms to genetic susceptibility to type 1 diabetes mellitus among Portuguese patients, we conducted a retrospective case-control study by analyzing four well-characterized *VDR* polymorphisms. We reported no evidence of allelic or genotypic association of the *Fok*I T>C (rs10735810), *Bsm*I A>G

Table 2 *VDR* haplotype frequencies in patients with type 1 diabetes mellitus and controls.

Haplotype[a]	Controls, *n* (%)	Patients, *n* (%)	Odds ratio (95% CI)	*p* value
*Fok*I/*Bsm*I/*Apa*I/*Taq*I	*n* = 223	*n* = 186		
C/G/G/T	77 (34.5)	66 (35.5)	1.04 (0.69-1.57)	0.840
C/A/T/C	56 (25.1)	52 (28.0)	1.16 (0.75-1.80)	0.516
T/G/G/T	50 (22.4)	35 (18.8)	0.80 (0.50-1.30)	0.371
T/A/T/C	16 (7.2)	10 (5.4)	0.74 (0.33-1.63)	0.458
C/G/T/T	11 (4.9)	13 (7.0)	1.45 (0.65-3.25)	0.378
T/G/T/T	5 (2.2)	4 (2.2)	0.96 (0.27-3.35)	1.000
C/A/T/T	4 (1.8)	1 (0.5)	0.30 (0.04-1.99)	0.382
C/A/G/T	3 (1.3)	2 (1.1)	0.80 (0.16-4.04)	1.000
T/A/G/T	1 (0.4)	0	0.00 (0.00-4.61)	1.000
C/A/G/C	0	2 (1.1)	∞ (0.63-∞)	0.206
T/A/G/C	0	1 (0.5)	∞ (0.31-∞)	0.455

CI = confidence interval.
[a] Haplotypes deduced from informative combinations of genotypes.

(rs1544410), *Apa*I G>T (rs7975232), or *Taq*I C>T (rs731236) SNPs of the *VDR* gene with type 1 diabetes mellitus in our population.

Such associations have been reported in populations from India [15], Taiwan [16], Germany [17–19], Hungary [14], Japan [20,21], the Netherlands [22], Croatia [23,24], Spain [25,26], and Chile [27]. However, no associations were detected in other populations from Brazil [28], Romania [29,30], Finland [30,31], Norway [30], the United States [30], and the United Kingdom [30].

The apparent discrepancies between this and other studies could be a result of the effect of ethnic differences related to the distribution of *VDR* polymorphisms in these populations, as well as to interactions with other genetic or environmental factors involved in the pathogenesis of type 1 diabetes mellitus. Human leukocyte antigen studies indicated that although the Portuguese are genetically related to Spaniards [32], they seem to have some ethnic-specific characteristics that distinguish them from other Europeans [32], and this may have contributed to the outcome of this study. Furthermore, because these polymorphisms, with the potential exception of the *Fok*I variant [33], have no known functional effects, the *VDR* itself may not be the disease-affecting locus, but rather a marker locus in linkage disequilibrium with the real disease locus, and the discrepant findings may reflect variable strengths of linkage disequilibrium in different populations.

In conclusion, our case–control study indicates that the four SNPs of the *VDR* gene studied are not associated with type 1 diabetes mellitus in the Portuguese population.

Acknowledgment

This work was supported, in part, by "Bolsa Dr. M. M. Almeida Ruas–Sociedade Portuguesa de Diabetologia/Novo Nordisk, em Diabetes (2003)."

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