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ORIGINAL ARTICLE

Family-based analysis of vitamin D receptor gene polymorphisms and type 1 diabetes in the population of South Croatia

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Abstract Type 1 diabetes mellitus (T1DM) is a disease characterised by the autoimmune destruction of insulin-producing pancreatic β cells. Vitamin D is a known immune system modulator and its effects are exerted via the vitamin D receptor (VDR). Several *VDR* gene single nucleotide polymorphisms (SNPs) have been commonly studied in relation to T1DM. The aim of this study was to evaluate the role of *VDR* gene variation in T1DM susceptibility by genotyping four SNPs (*Fok*I-rs10735810, *Taq*I-rs731236, *Bsm*I-rs1544410, and *Tru9*I-rs757343) in 160 case–parent trio samples from the population of South Croatia. We observed overtransmission of *Tru9*I allele G and undertransmission of the *Tru9*I-*Bsm*I A-A haplotype from parents to affected children ($P = 0.032$, $P = 0.002$, respectively). These results indicate a possible role of the *VDR* gene in T1DM aetiology. In conclusion, this family-based study presents some evidence of association of specific *VDR* gene variants with T1DM in the population of South Croatia.

Keywords Type 1 diabetes · Vitamin D receptor · TDT · Polymorphism · Genetic epidemiology · Croatia

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Introduction

Type 1 diabetes mellitus (T1DM) has a strong genetic component. The most important genetic factors for determining the risk of developing T1DM reside in the human leucocyte antigen (HLA) class II loci, but several other gene regions have also been identified: a region 5′ to the *INS* gene, *CTLA4* gene, *PTPN22* gene, *IL2RA* gene and *IFIH1* region (Rich et al. 2006; Smyth et al. 2006). The most recent genome-wide association study of T1DM identified another four regions: 12q24, 12q13, 16p13 and 18p11 (Todd et al. 2007).

So far, a large number of studies have suggested that the vitamin D receptor gene (*VDR*) is involved in the pathogenesis of T1DM. Among different roles in the organism, vitamin D acts as a modulator of the immune system by promoting monocyte differentiation and inhibiting lymphocyte proliferation and secretion of several cytokines (Haussler et al. 1998; Uitterlinden et al. 2004). Maternal intake of vitamin D through food during pregnancy may protect offspring against the appearance of islet autoimmunity (Fronczak et al. 2003). Oral administration of the hormonally active form of vitamin D completely protects nonobese diabetic mice (NOD) mice from T1DM (Zella et al. 2003). In addition, treatment of adult NOD mice with a vitamin D analogue markedly reduced T1DM development (Giarratana et al. 2004). The effects of vitamin D are mediated by the VDR protein, a nuclear receptor that acts

as a ligand-activated transcription factor. Therefore, sequence variation in the *VDR* gene may be related to T1DM (Valdivielso and Fernandez 2006).

The *VDR* gene, located at chromosome 12q12-q14, has several frequently studied single nucleotide polymorphisms (SNP). These are *Fok*I (rs10735810) G/A change in exon 2, *Taq*I (rs731236) T/C change in exon 9, *Bsm*I (rs1544410) G/A and *Tru9*I (rs757343) G/A changes both in intron 8 (Guo et al. 2006). *Fok*I occurs at the first start codon in exon 2 and changes the translation initiation site, resulting with three amino acids shorter truncated protein, considered to be more active than the wild type (Uitterlinden et al. 2004). It is not yet known if intronic *Bsm*I and*Tru9*I SNPs and a synonymous exonic *Taq*I SNP have any functional effect (Valdivielso and Fernandez 2006). Nevertheless, these three SNPs can be useful in association studies as markers to some other truly functional allele elsewhere within this gene region (Uitterlinden et al. 2004).

VDR gene polymorphisms were largely studied in the last decade within different world populations. The results reported in those studies showed inconsistency, and Guo et al. evaluated those reports in a meta-analysis of the four *VDR* gene SNPs (*Fok*I, *Bsm*I, *Apa*I and *Taq*I) but not *Tru9*I (Guo et al. 2006). The authors found no evidence for an association between *VDR* gene polymorphisms and T1DM risk. Nejentsev et al. studied 98 SNPs within the *VDR* gene (including four SNPs) in a larger group of 3,763 families and found no evidence of association with T1DM (Nejentsev et al. 2004). Recently, Ramos–Lopez et al. reported a protective role of several VDR haplotypes in T1DM (Ramos-Lopez et al. 2006). Also, Mimbacas et al. found *Fok*I SNP to be associated with T1DM in a Uruguayan population (Mimbacas et al. 2007).

This family-based study is an extension of our population-based case-control studies of the *VDR* gene variants (Skrabic et al. 2003; Zemunik et al. 2005). Previously, we found combined genotypes BBAAtt (Skrabic et al. 2003), *Fok*I SNP and several haplotypes (Zemunik et al. 2005) to be associated with susceptibility to T1DM. The aim of this study was to analyse the transmission of *Fok*I, *Taq*I, *Bsm*I and *Tru9*I SNPs in case–parent trio samples and to evaluate their association with susceptibility to T1DM in the population of South Croatia.

Materials and methods

Subjects

We typed 132 parent–offspring trios, 20 parent–offspring duos, seven families with two affected children and one family with three affected children from the population of South Croatia. Each proband was ascertained with T1DM according to the World Health Organisation criteria. Gender 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 ± standard deviation (SD)]. This study was approved by the ethics committee, and informed consent from was obtained from patients and their parents prior to blood sampling.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Genotypes for four *VDR* SNPs (*Fok*I-rs10735810, *Taq*I-rs731236, *Bsm*I-rs1544410 and *Tru9*I-rs757343) were identified by polymerase chain reaction (PCR) followed by restriction fragment-length polymorphism (RFLP) (Ban et al. 2001; Chang et al. 2000; Ye et al. 2000). The digested fragments were separated in 8% polyacrylamide gels (*Fok*I and *Tru9*I) and 3% agarose gels (*Taq*I and *Bsm*I) and visualised by ethidium bromide staining. Genotypes were determined according to the presence or absence of restriction site, and alleles were designated respective to actual base change according to the dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/).

As a measure of quality control (QC), all samples were checked for gender status using a fluorescence-based competitive allele-specific assay (Kaspar, Kbioscience, UK). The assay investigates the single base difference between a homologous exon of the *ZFX* and *ZFY* genes and is diagnostic for sex chromosome (assay details are available from the authors on request).

Statistical analysis

Prior to association analysis, we performed QC of the obtained genotypes. As a part of QC, data was tested for Mendelian inheritance and missing genotypes using Plink (Purcell et al. 2007). Hardy-Weinberg equilibrium (HWE) in healthy parents was tested using Pedstats (Wigginton and Abecasis 2005). Minor allele frequencies (MAF) were compared with phase II HapMap (http://www.hapmap.org) MAF from the CEU population (The International HapMap Consortium 2003). Single-point and multipoint association analyses were carried out using Plink and Unphased (Dudbridge 2003) implementations of the transmission disequilibrium test (TDT). Power calculation for every investigated SNP was performed using Quanto (Gauderman 2003). To obtain empirical *P* values, 10,000 permutations were run for each analysis, and permutation *P* values less than 0.05 were considered nominally significant.

Results

Gender-control genotyping identified six discrepancies when compared with database information. These six individuals and their families were excluded from further analysis. No Mendel errors were found in the rest of the obtained genotyped trios. *Fok*I and *Tru9*I SNPs were genotyped for every sample, and the percentage of missing genotypes per *Taq*I and *Bsm*I SNPs were 1.3% and 0.8%, respectively. *Bsm*I SNP was out of HWE, and *Taq*I was at the limit of the significance in healthy parents ($P = 0.0026$ and $P = 0.0282$, respectively). MAF in healthy parents were concordant with phase II HapMap frequencies from the CEU (European descent) population. After QC analysis, 154 cleaned-up families were submitted to further analyses.

TDT single-point analysis detected nominally significant overtransmission of the *Tru9*I common allele G from parents to affected child [$P = 0.032$, odds ratio (OR) = 1.73, 95% confidence interval (CI) 1.041–2.904, permutation 10,000]. Results of TDT analysis are shown in Table 1. Exhaustive TDT multipoint search for haplotypic effects observed an association with *Tru9*I-*Bsm*I haplotypes ($P = 0.005$, permutation 10,000). Specifically, the *Tru9*I-*Bsm*I A-A haplotype was undertransmitted from parents to affected offspring ($P = 0.002$) (Table 2). This study had 80% statistical power to detect (at $\alpha = 0.05$) an effect of OR = 1.6 for *Fok*I, *Taq*I and *Bsm*I and 65% power for OR = 1.73 for *Tru9*I, assuming an additive model.

Discussion

In this study, we found some evidence of association of *VDR* gene *Tru9*I polymorphism and *Tru9*I-*Bsm*I haplotype with T1DM in the population of South Croatia. However, this study has several limitations. First, our sample size was not big enough to detect an effect of OR = 1.73 for *Tru9*I SNP (we had 65% statistical power). Second, we performed a number of tests and did not correct for multiple testing. However, for every significant result, we ran 10,000 permutations and reported a permutation *P* value. Third, we observed a deviation of *Bsm*I SNP from HWE. This deviation could reflect on the positive results of *Tru9*I-*Bsm*I haplotype we found. Nevertheless, the possibility of true finding should not be excluded.

There are several positive aspects of this study. It investigates four *VDR* gene variants in the T1DM families from South Croatia for the first time. It contributes to the overall knowledge of the relation of the *VDR* gene and T1DM. It is designed to be compatible for comparisons between different populations and will be useful for future meta-analysis of the *VDR* gene.

We observed a deviation in transmission of *Tru9*I alleles (overtransmission of allele G; i.e. undertransmission of minor allele A) from parents to affected offspring. The *Tru9*I MAF of the affected probands (0.099), due to its undertransmission, is expectedly lower than the MAF of the HapMap for CEU populations (0.133). But this difference is not significant ($P = 0.340$). We also observe a pattern of undertransmission of *Tru9*I-*Bsm*I A-A haplotype. All these results support the idea of a possible protective role of the *Tru9*I minor allele in T1DM aetiology.

We have already analysed *VDR* gene variants with T1DM in two population-based case-control studies (Skrabic et al. 2003; Zemunik et al. 2005). Our results presented here were unable to confirm previously reported single-point associations. In multipoint analysis, we could not detect significant associations of previously reported haplotypes (BatU, FbATu and fBATU) with T1DM (Zemunik et al. 2005). However, in this family-based study, we observe the same trend towards protectiveness;

Table 2 Transmission of *Tru9*I-*Bsm*I haplotypes in 154 parent–offspring trio families

Haplotype	*T*	*U*	χ^2	*P* value
A-A	4.216	18.86	9.291	0.002
G-A	73.78	56.14	2.396	0.122
A-G	18.78	21.14	0.1393	0.709
G-G	62.22	62.86	0.003295	0.954

Numbers of transmitted (*T*) and nontransmitted (*U*) haplotypes

Table 1 Transmission disequilibrium analysis of minor allele of four vitamin D receptor gene single nucleotide polymorphisms (*VDR* SNPs) in 154 parent–offspring trio families

SNP	CA	MA	MAF	*T*:*U*	OR	*L*95	*U*95	χ^2	*P* value
*Taq*I (rs731236)	T	C	0.447	65:54	1.204	0.84	1.73	1.017	0.313
*Tru9*I (rs757343)	G	A	0.099	23:40	0.58	0.34	0.96	4.587	0.032
*Bsm*I (rs1544410)	G	A	0.494	57:54	1.06	0.73	1.53	0.081	0.776
*Fok*I (rs10735810)	G	A	0.407	74:73	1.01	0.73	1.40	0.007	0.934

CA common allele, *MA* minor allele, *MAF* minor allele frequencies in type 1 diabetes mellitus (T1DM) children, *T:U* copies of the minor allele transmitted (*T*) and nontransmitted (*U*) from heterozygous parents to affected offspring

i.e. we observed relative undertransmission of similar haplotypes from parents to affected children (BtU, FbTu and fBTU; i.e. GCG, GATA and AGTG, respectively) but without reaching significant difference.

Yet published findings on association of several SNPs in the *VDR* gene with a risk of developing T1DM have been conflicting. Guo et al. tried to evaluate these findings in a meta-analysis of *Fok*I, *Taq*I, *Bsm*I and *Apa*I polymorphisms and found no evidence of association (Guo et al. 2006). However, they did not analyse the *Tru9*I variant. Nejentsev et al. developed a *VDR* gene SNP map and tested all common variation of this gene, including four variants from our study, in five different populations. Their results indicated no major effect of common variation in the *VDR* gene and susceptibility to T1DM (Nejentsev et al. 2004). Recently, Ramos-Lopez et al. investigated 11 polymorphisms, also including the same four polymorphisms from our study, in German families. They found several *VDR* gene haplotypes to be negatively associated with T1DM (Ramos-Lopez et al. 2006). Mimbacas et al. investigated three *VDR* SNPs and found *Fok*I to be an indicator for susceptibility to T1DM in a Uruguayan population (Mimbacas et al. 2007). Recent reports remain equivocal, and a replication of the study in a bigger sample is needed.

The incidence of T1DM has large geographical variations, which might be caused by differences in frequencies of genetic markers among different populations (Angel et al. 2004). Findings from our study might indicate the differences in the genetic background between studied populations and/or could point out linkage to truly causal variants somewhere else within this chromosomal region (Valdivielso and Fernandez 2006).

Our study investigated four *VDR* gene SNPs in several T1DM families of South Croatia. We found evidence of association of *Tru9*I SNP and *Tru9*I-*Bsm*I haplotype with T1DM. Further work should focus on the synthesis of published results and analyses in a larger sample to clarify the role of the *VDR* gene as T1DM marker.

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