CLINICAL STUDY

Gender-specific association of vitamin D receptor polymorphism combinations with type 1 diabetes mellitus

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Abstract

Objective: Recent data have indicated the significance of vitamin D receptor (VDR) polymorphisms in type 1 diabetes mellitus (T1DM). We have studied the association of five known restriction enzyme polymorphisms of the VDR gene in patients with T1DM.

Design and Methods: One hundred and seven children with T1DM (T1DM for 5 years; age, 1–14 years; boys/girls, 57/50; body mass index, 17.0 ± 2.3 kg/m²; haemoglobin A_{1c} (HbA_{1c}), 7.87 ± 1.05) and 103 healthy subjects were enrolled. The VDR polymorphisms ApaI, BsmI, FokI, TaqI and Tru9I ('a', 'b', 'f', 't' and 'u' alleles respectively) were investigated.

Results: The 't' and 'T' alleles miss the Hardy–Weinberg equilibrium ($P < 0.01$) in control and diabetic populations; we therefore excluded this polymorphism from further analysis. We did not find a difference in the allele prevalence in T1DM patients and controls of any of the five polymorphisms. However, when the 'b', 'a' and 'u' alleles were simultaneously compared in girls, there was a significantly higher prevalence in patients with diabetes compared with controls ('b' + 'a' + 'u' present/absent: healthy, 0/53; diabetic, 13/37; $P < 0.005$). In boys the prevalence of 'b' + 'a' + 'u' genotype was similar in T1DM and controls.

Conclusions: The impact of the 't' allele cannot be investigated in this study population. Not a single VDR polymorphism increases the susceptibility to T1DM. The common presence of the 'b', 'a' and 'u' alleles greatly increases the probability of T1DM in girls.

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Introduction

Dietary vitamin D supplementation has recently been associated with reduced risk of type 1 diabetes (T1DM) in a large-scale study (1). *In vitro* vitamin D acts as an immunosuppressive agent. It reduces lymphocyte activation, proliferation and cytokine production (2, 3) and might influence the immune-mediated destruction of insulin-producing β -cells of the pancreas. Moreover, vitamin D deficiency leads to impaired insulin secretion, which is reversible by 1,25-dihydroxyvitamin D₃ administration. This process has been described in human and other animal studies (4–6). The pleiotropic biological actions of vitamin D are mediated by the vitamin D receptor (VDR) (7, 8). The importance of vitamin D is further supported by the finding that pancreatic β -cells express VDR (9).

The function of the VDR gene is influenced by several genetic polymorphisms. The schematic structure of the VDR gene and the localization of restriction sites are

presented in Fig. 1. The polymorphic FokI site in exon 2 results in an alternative transcription initiation site, leading to the addition of three amino acids to the VDR protein in individuals with the 'f' allele (10). BsmI ('b'), ApaI ('a') and Tru9I ('u') sites are in introns, but alterations in intronic sequence may influence protein expression. A silent T to C substitution at position 1055 in exon 9 creates the TaqI restriction site (the 't' allele) (11, 12). According to specific data, VDR polymorphisms are associated with plasma calcitriol levels (13), parathyroid calcium response and calcium sensor protein (14–16), and urinary calcium excretion (17). Polymorphisms do have an impact on hyperparathyroidism (13, 14, 18, 19), Graves' disease (20), bone mass and turnover (12, 21), primary biliary cirrhosis (22), oncogene expression and rectal cancer prognosis (23).

The effects of VDR polymorphisms on insulin secretion have been reported (24). Correlation between the BsmI polymorphism and type 2 diabetes (T2DM)

Figure 1 Schematic structure of the human VDR gene. The exons are indicated by numbers. The positions of the restriction sites investigated are also shown (11, 12, 18, 29–32).

has also been documented (25). Other studies observed no correlation between T2DM and BsmI, TaqI, ApaI and Tru9I polymorphisms of the VDR gene (26). Combinations of VDR BsmI, ApaI and TaqI polymorphisms increased the susceptibility to T1DM in Germans (11) and in Indian Asians (27). However, no data are available about the Tru9I polymorphism in T1DM.

We have investigated the prevalence of each known VDR polymorphism as well as their association with clinical characteristics in children with T1DM.

Patients and methods

Patients

One hundred and seven unrelated Caucasian children with T1DM attending the First Department of Paediatrics, Semmelweis University, Budapest, Hungary, were enrolled on the study. The diagnosis of T1DM was established at least 5 years prior to the investigation. The boy to girl ratio of T1DM probands was 57/50. To characterize our diabetic population, we recorded age at the onset of diabetes, body mass index (BMI), microalbuminuria and serum haemoglobin A_{1c} (HbA_{1c}). All patients with diabetes were islet cell autoantibodies (ICA) positive (10 < juvenile diabetes foundation units (JDFU)). Age at the onset of diabetes was between 1 and 14 years in the diabetic group, with an average of 5.8 ± 3.2 years. The average BMI of all patients was 17.0 ± 2.3 kg/m². BMI values were related to the age-matched percentile values. Each child's BMI was within the 25–75 percentile interval. Microalbuminuria was present in 34% of patients, the serum HbA_{1c} level was 7.87 ± 1.05 . No differences were detected between boys and girls.

Reference values for the prevalence of each genetic variant were obtained from 103 healthy blood donors (53 men and 50 women). The advantage of this population is the extremely low risk of development of T1DM. They were not screened for ICA autoantibodies.

However, each donor filled out a detailed questionnaire concerning health risk factors before blood donation and none of them indicated the presence of T1DM in first-degree relatives. They were all from Budapest, as were the diabetic patients.

At the beginning of sample collection the informed consent of parents was obtained for the use of samples from their children for diagnostic and scientific purposes. At the beginning of data processing all samples received a unique three digit number, and during further analyses this number was used without referring to the patient's name. An independent institutional ethical committee approved the study.

DNA isolation

Blood was collected in EDTA-K₃ tubes and stored at -20°C until DNA extraction. DNA was purified by standard proteinase K digestion, phenol–chloroform extraction and ethanol precipitation (28).

Genotyping

DNA was amplified with a standard PCR technique. After initial denaturation for 4 min at 94°C , samples were subjected to 35 cycles of amplification, consisting of a 30-s denaturing phase at 94°C , a 30-s annealing phase (see temperature below) and a 30-s extension phase at 72°C . A 4-min 72°C hold was the final step of the programme. After restriction, the end products were electrophoresed on a 2% agarose gel containing 0.4 mg/l ethidium bromide. The polymorphism was documented by photographing under UV illumination. We used an internal control for verification of complete digestion. A random subset ($\sim 10\%$ of samples) was repeatedly measured to verify the results. Upper case letters indicate the absence of the cut site, whereas lower case letters indicate its presence. Letters for the alleles are the first letters of the restriction enzyme, except for Tru9I, where 'u' is used.

FokI polymorphism

The sense primer: 5-AGC TGG CCC TGG CAC TGA CTC TGC TCT-3, and the reverse primer: 5-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3 (29) were used at an annealing temperature of 60°C . Five units of FokI restriction enzyme (Promega Corp., Madison, WI, USA) and 1.5 μl buffers were added to 10 μl of the PCR product. The overnight restriction was at 37°C according to the supplier's recommendation. The FokI alleles are designated 'F' and 'f'.

The amplified sequence starts at position 11963 in Genbank (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide>) accession no. AC004466 (30), the amplified fragment is 265 bp in length. The restriction enzyme for FokI polymorphism binds at position 12 019 (GGATG(N)⁹).

ApaI and TaqI polymorphisms

For the detection of ApaI and TaqI polymorphisms the sense primer: 5-CAG AGC ATG GAC AGG GAG CAA-3 and the reverse primer: 5-GCA ACT CCT CAT GGC TGA GGT CTC-3 were used at an annealing temperature of 70 °C (31). The digestion with ApaI (GGGCC[^]C; F Hoffmann-La Roche Inc., Nutley, NJ, USA) was performed with 10 U enzyme and 10 µl PCR product at 30 °C for 4 h. The ApaI alleles are designated 'A' and 'a'. From the enzyme TaqI (T[^]CGA; MBI Fermentas Inc., St Leon-Rot, Germany) 3 U was added to the amplified mixture, and digestion was at 65 °C for 4 h. The TaqI alleles are designated 'T' and 't'.

The 740 bp length amplified sequence starts at position 111403 and ends at position 112143 in Genbank accession no. AC004466. After digestion with ApaI, a 530 bp and a 210 bp length fragment can be detected. TaqI has two binding sites on the PCR product, one produces a 495 bp and a 245 bp fragment, and the other is at the single nucleotide polymorphism site in the presence of which the 495 bp fragment will be cut into a smaller 290 bp piece and a 205 bp piece.

BsmI polymorphism

The sense primer: 5-AGT GTG CAG GCG ATT CGT AG-3 and the reverse primer: 5-ATA GGC AGA ACC ATC TCT CAG-3 were used at an annealing temperature of 62 °C. For the restriction, 5 U of the BsmI enzyme (GAA-TGCN[^]; Qbiogene Inc., Heidelberg, Germany) was used at 65 °C for 4 h (18). The BsmI alleles are designated 'B' and 'b'.

The amplified sequence is between positions 45 007–45 198 and 110 543–110 734 in Genbank accession no. AC004466, the amplified fragment is 191 bp in length and after successful digestion a 115 bp and a 76 bp line can be seen. One of the BsmI cut sites (GAACTGg[^]) is at 45 123, the other at 110 658.

Tru9I polymorphism

The sense primer: 5-TGA GGT TTC TTG CGG GCA GGG TA-3 and the reverse primer: 5-CAG GGC CGC CCC TCT TTG GA-3 were used at an annealing temperature of 72 °C. Five units of the Tru9I enzyme (Promega Corp.) digested the PCR product for 4 h at 65 °C. We designed primers using the Primer3 software (http://www-genome.wi.mit.edu/genome_software/other/primer3.html) on the VDR Genbank sequence AC004466 (30, 32). The amplified sequence starts at position 90 260 in Genbank accession no. AC004466, the amplified fragment is 212 bp in length. The Tru9I alleles are designated 'U' and 'u'.

Statistical analyses

Hardy–Weinberg equilibrium was calculated to evaluate the relationship between gene frequencies and genotype frequencies. Chi-square tests and Fisher's exact test were used to compare prevalence of genetic polymorphisms in T1DM and in controls. Odds ratios were computed when data were significantly different. For linkage and distribution calculations the Arlequin software (<http://anthropologie.unige.ch/arlequin/>) was used. The level of statistical significance was set at $P < 0.05$.

Results

The prevalence of the 'f', 'b', 'a' and 'u' alleles are in the Hardy–Weinberg equilibrium. The 't' and 'T' alleles miss the Hardy–Weinberg equilibrium ($P < 0.0001$). Based on these results we excluded the TaqI polymorphism from further analysis (see Table 1).

The prevalence of 'f', 'b' and 'a' alleles did not differ between boys and girls, either in T1DM or control subjects. The homozygous genotype 'uu' was not present in T1DM and controls. Clinical characteristics and VDR alleles were not associated.

The alleles 'b' and 'a', 'b' and 'u' were in linkage disequilibrium in control and T1DM subjects. The alleles 'a' and 'u' were in linkage disequilibrium only in controls (See Table 2). The 'f' allele was not associated with any of the other alleles.

The prevalence of studied polymorphisms did not differ between healthy and diabetic subjects (see Table 1).

Table 1 Prevalence of VDR gene polymorphisms and alleles in control and diabetic subjects.

	Control	Allele	Diabetic	Allele
FokI				
FF	0.33		0.28	
Ff	0.46		0.55	
ff	0.20	'f' = 0.43	0.17	'f' = 0.44
BsmI				
BB	0.16		0.18	
Bb	0.50		0.43	
bb	0.33	'b' = 0.58	0.39	'b' = 0.60
TaqI				
TT	0.41		0.41	
Tt	0.26		0.32	
tt	0.31	't' = 0.45	0.25	't' = 0.41
ApaI				
AA	0.31		0.22	
Aa	0.42		0.52	
aa	0.25	'a' = 0.46	0.25	'a' = 0.51
Tru9I				
UU	0.81		0.78	
Uu	0.18		0.21	
uu	0	'u' = 0.09	0	'u' = 0.10

The polymorphisms FokI, BsmI, ApaI and Tru9I ('f', 'b', 'a' and 'u' respectively) are in Hardy–Weinberg equilibrium. The difference between the expected and measured distributions at the TaqI ('t') polymorphism are significant ($P < 0.0001$).

Table 2 Linkage disequilibrium values between the restriction sites BsmI, Apal and Tru9I ('b', 'a' and 'u' respectively) in control and diabetic subjects.

	D'	Chi-square	P
Control			
'b' × 'a'	0.86	23.12	<0.001
'b' × 'u'	-0.75	5.04	<0.05
'a' × 'u'	0.60	7.39	<0.01
Diabetic			
'b' × 'a'	0.78	44.04	<0.001
'b' × 'u'	-0.76	4.14	<0.05
'a' × 'u'	0.12	1.36	n.s.

Correlation with the 'f' allele was not observed. D', standardized disequilibrium values; n.s., not significant.

No single allele was associated with T1DM. However, the prevalence of 'Uu' was higher in girls with T1DM compared with control subjects (girls/boys: control 7/18 vs T1DM 16/5; $P < 0.01$).

The number of girls carrying the alleles 'b', 'a' and 'u' simultaneously was higher in T1DM than in controls ('b' + 'a' + 'u' present/absent: diabetic 13/37; healthy, 0/53; $P < 0.005$). The clinical characteristics and age at the onset of T1DM did not differ between T1DM girls with or without 'b' + 'a' + 'u'. In boys such a phenomenon was not present ('b' + 'a' + 'u' present/missing: healthy, 1/52; diabetic, 1/56; not significant) (see Table 3).

Discussion

The prevalence of 'f', 'b', 'a' and 'u' alleles in healthy controls was similar to data reported previously in Japanese (29) and Caucasian populations (18, 32). The prevalences were similar to those in our T1DM patients and control subjects.

Our measured 't' allele frequencies were different from those found by Ongphiphadhanakul *et al.* (17) in Thailand. However, the distributions of the 't' and

'T' alleles were not in Hardy–Weinberg equilibrium. Therefore, the association between the 't' allele and T1DM was not analyzed in our study. One can speculate why the Hardy–Weinberg criteria are not fulfilled in a healthy population. The Hungarian population's unique genetic background might be one of the possible explanations (33).

Previous studies reported linkage between the alleles 'a', 'b' and 't' (34, 35). Uitterlinden *et al.* (35) found that, after constructing molecular haplotypes across this area of the VDR gene, the most frequent haplotypes in Caucasians are 'baT' (50%), 'Bat' (40%) and 'bAT' (10%). They also suggested that the variation of the degree of linkage disequilibrium between restriction fragment length polymorphism loci in the studied population might influence the degree of association between alleles and characteristics (36). Although we could not analyse the 't' allele, we have detected a strong linkage disequilibrium between the 'b', 'a' and 'u' alleles. This suggests that their inheritance is not independent in our study population. The close location of these polymorphisms might be an explanation for the stability of this linkage, but the background of these combinations will need further investigation. The linkage between the 'b' and the other alleles suggests that these polymorphisms could possibly be responsible for the clinical associations previously associated with BsmI but where other polymorphisms were not associated (11, 25, 27).

Pani *et al.* (11) reported an increased susceptibility to T1DM in Germans with combinations of VDR polymorphisms. In their study, the 'At', 'Bt' and 'BAT' alleles presented an increased risk, whereas 'AT' and 'at' were protective. Since we could not analyze the effect of 't' alleles in our population, we cannot confirm or reject their observations.

During our study we found a strong association between the carrier state of the 'b' + 'a' + 'u' alleles and the presence of T1DM in girls. Our results suggest that this allele combination presents an important risk for T1DM, although it does not influence the other clinical parameters of T1DM studied. This novel finding suggests a gender-specific difference. Other publications also report association between gender and the consequences of gene polymorphisms (37). Polymorphism in the hormone-sensitive lipase is associated with markers of insulin sensitivity in women but not in men (38) and VDR polymorphisms are associated with sex-dependent growth (39). However, the significance of this finding is hard to appreciate since the disease generally affects males and females equally, raising the question as to whether this finding is related to a specific feature of the selected Hungarian population rather than a true association with the disease. Confirmation of the association with other genetic approaches, for example using family studies, would provide more convincing evidence.

Table 3 Prevalence of haplotypes in control and diabetic subjects: the presence of polymorphisms BsmI, Apal and Tru9I ('b', 'a' and 'u' respectively).

	Control		T1DM	
	U	u	U	u
Males				
BA	0.16	—	0.17	0.02
Ba	0.02	—	0.05	—
bA	0.02	0.10	—	0.07
ba	0.66	0.02	0.67	0.02
Females				
BA	0.25	—	0.16	—
Ba	—	—	0.02	—
bA	—	0.06	—	0.07
ba	0.69	—	0.47	0.29*

* $P < 0.01$ compared with controls.

Evaluation of our data is further complicated by the fact that there are two restriction sites for BsmI ('b') in the VDR gene. Studying the Genbank sequence we noticed that a large DNA sequence – containing the BsmI restriction site and both sense and reverse primer binding sites – is present in a repetitive area twice in the VDR gene. The lengths of the amplified sequences are identical and, using our method, it cannot therefore be decided whether there are one or two BsmI restriction sites in heterozygous individuals. This characteristic of the VDR gene has not been mentioned in the interpretation of previous data (11, 13, 18, 22, 23). Further investigation is needed to clarify whether only one or both of the two 'b' alleles are present in diabetic 'b' + 'a' + 'u'-positive girls who are heterozygous for the 'Bb' alleles.

Our report is the first one to collect data for frequencies of all five known restriction sites of the VDR gene in healthy and T1DM Caucasian populations. Our results suggest that the common presence of the cut sites for the BsmI, ApaI and Tru9I polymorphism of the VDR gene in the Hungarian population greatly increases the probability of T1DM in girls but not in boys. These results should also be evaluated for other ethnic groups.

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