

CLINICAL STUDY

Vitamin D receptor polymorphisms: no association with type 1 diabetes in the Finnish population

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Abstract

Objective: The effect of polymorphisms of the vitamin D receptor (VDR) gene on susceptibility to type 1 diabetes has recently been investigated extensively. Several findings on positive disease associations have been observed and, in addition, a protective effect of vitamin D supplementation has been reported.

Design: We studied the effect of three vitamin D receptor gene polymorphisms (VDRA, VDRB, VDRF) on susceptibility to type 1 diabetes in a large case-control series (more than 2000 controls and about 1000 patients) from the Finnish population.

Methods: A combination of case-control and affected-family based approaches was used. Subjects were genotyped for VDRA (ApaI), VDRB (BsmI) and VDRF (FokI) single nucleotide polymorphisms using a minisequencing reaction.

Results: A few borderline significant associations were observed with both approaches used. In the case-control association analyses we found significant differences between cases and controls in frequencies of VDRB ($P = 0.024$, all subjects and $P = 0.016$, HLA DQB1*0302-positive subjects) and VDRF ($P = 0.0063$, Turku cohort). In the total family set a decreased (39.3%) transmission of the VDRA-VDRB-VDRF haplotype 1-1-2 and an increased (60.3%) transmission of haplotype 2-1-2 to sons was seen ($P = 0.0059$ and $P = 0.024$ respectively). Transmission of the haplotype 2-2-1 to daughters was decreased (37.6%, $P = 0.022$). Interestingly, we also observed significant differences in allele frequencies of the polymorphisms studied between populations from three different regions in Finland.

Conclusions: All these differences disappeared after correction for multiple testing. We conclude that the single nucleotide polymorphisms analysed are unlikely to be associated with type 1 diabetes in the Finnish population.

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Introduction

Type 1 diabetes (T1D) is an immune-mediated disorder where clinical disease develops as a result of interactions between genetic factors and the environment. The destruction of the insulin-producing β -cells is preceded by insulinitis characterised by T-lymphocyte infiltration into the pancreatic islets (1).

While HLA class II genes (DQB1, DQA1, DRB1) on chromosome 6p comprise the strongest genetic component of the disease, there are a number of other loci reported to be linked to and associated with T1D (2–5). In addition to HLA genes, the insulin gene region at chromosome 11p15 is also regarded as

a confirmed susceptibility locus for T1D (3, 6). There are also other putative loci not identified by genome-wide scans that are potentially good candidates and may contribute to disease susceptibility.

At least two epidemiological studies have suggested that vitamin D supplementation decreases the risk of T1D. The EURODIAB study group showed a protective effect in a large international multicentre survey (7) using data collected from seven different centres, while Hyppönen *et al.* obtained similar findings in a birth-cohort study in northern Finland (8). Vitamin D is also a potent regulator of cell growth and differentiation and thus a candidate for cancer regulation. In addition, some cancer cells express vitamin D receptor

molecules and a number of studies indicate that vitamin D or its analogues can inhibit cancer proliferation (9, 10).

It has been demonstrated in experimental studies that vitamin D prevents or markedly suppresses the development of various autoimmune diseases in animal models. In addition to NOD mice, in which vitamin D and its analogues prevent insulinitis, administration of vitamin D was found to protect against e.g. autoimmune thyroiditis (11), experimental rat nephritis (12), experimental encephalomyelitis (13, 14) and rheumatoid arthritis (15). Vitamin D has been shown to be an effective immunosuppressant via inhibition of lymphocyte activation and cytokine production (16–18). These effects might be mediated through the regulatory function of the active form of vitamin D on T-cell-mediated immunity.

The biological effects of vitamin D are delivered through the vitamin D receptor gene, VDR (OMIM No 601769, at chromosome 12q12-q14). The gene encodes a polypeptide that binds 1,25-dihydroxy-calciferol (activated vitamin D) and interacts with target nuclei to produce a variety of effects. VDR has been shown to be present in significant concentrations in T lymphocytes and macrophages (19), and some of its polymorphisms have recently been found to be associated with several autoimmune diseases in man, such as psoriasis (20), Grave's disease (21, 22), Addison's disease (23) and T1D (24–31). In addition, the VDRF and VDRB single nucleotide polymorphisms (SNPs) were suggested to have an effect on GAD65-autoantibody positivity in T1D (30) and on the onset pattern of the disease (31), respectively, in Japanese case-control analyses.

To confirm the effect of VDR gene polymorphisms on the risk of T1D we used a combination of case-control and affected-family based analyses in a large series to study the possible association of three VDR SNPs (VDRA, VDRB and VDRF, also commonly known as ApaI, BsmI and FokI restriction sites) with T1D in the Finnish population.

Subjects, materials and methods

All the patients ($n = 1064$, 577 males) had classical clinical presentation of T1D with hyperglycaemia and ketosis and were diagnosed under the age of 15 years

according to the World Health Organisation criteria. Autoantibodies to islet cells were analysed at diagnosis and found to be positive in most cases. The study was approved by the Ethics Committee of the Turku University Hospital and informed consent was obtained from the participating subjects and/or their parents.

Three SNPs (VDRA, VDRB and VDRF) were genotyped in children with T1D ($n = 944$, 1064 and 985 respectively), and in healthy infants ($n = 2379$, 2837 and 2356 respectively) consecutively enrolled in the genetic screening programme of the Finnish Diabetes Prediction and Prevention (DIPP) study. This control material well represented the background population. Most of the samples were taken from patients diagnosed and treated in three different centres: Turku representing southwest Finland, Tampere in the central part of the country and Oulu in northern Finland (see Table 2 for the detailed distribution of the samples). In addition, parents of 544 patients were genotyped to study the transmission of alleles by means of the transmission disequilibrium test (TDT).

All three SNPs were genotyped using a minisequencing kit (SnuPe Kit, Amersham Biosciences, Amersham, Bucks, UK) according to the manufacturer's instructions. The method was based on a reaction in which a purified PCR product (ExoSAP-IT, USB Corporation, Cleveland, OH, USA, 2 μ l/5 μ l PCR product) was used as a template, only one primer was added and only labelled ddNTPs were used. The primer was designed so that the SNP was the next base from the 3'-end of the primer. Purified minisequencing products (Shrimp Alkaline Phosphatase, USB Corporation, 1 U/10 μ l SNP reaction) were analysed using a MegaBACE1000 sequencer (Amersham Biosciences, CA, USA). Primer sequences and the length of PCR products are listed in Table 1.

A combination of case-control and affected-family approaches were used. Allele and genotype frequencies were obtained by direct counting. The chi-square test was used for the calculation of statistical significance and the level was set at a P value of less than 0.05. The transmission disequilibrium test was carried out using the TDTPHASE software (32). Transmissions to sons and to daughters as well as from fathers and from mothers were analysed separately to identify the possible sex-specific effect in transmissions. In order to increase the statistical power by reducing the

Table 1 Primer sequences used in the study and length of PCR products.

	PCR	Minisequencing
VDRA 384 bp	5'-CAGAGCATGGACAGGGAGCA-3' 5'-CTTGGCATAGAGCAGGTGGC-3'	5'-GTGGATTGAGCAGTGAGG-3'
VDRB 758 bp	5'-CCATTGTCTCTCACAGCCG-3' 5'-AGCCCATCTCCATTCTTGA-3'	5-GAGCCTGAGTATTGGGAATG-3'
VDRF 147 bp	5'-GGCCCTGGCACTGACTCT-3' 5'-GGCTCGGTCTCCACACAC-3'	5'-GCCGCCATTGCCTCC-3'

possible heterogeneity in the patient data, we also stratified the data according to the patients' HLA DQB1 genotypes (*02/*0302, *0302/x, *02/y, others; $x \neq *02$, $y \neq *0302$) and age at diagnosis (0–4 years, 5–9 years, 10–14 years). Male and female patients were also analysed separately in case-control comparisons.

In addition, three different control sets (Turku, Tampere, Oulu) were compared with each other to analyse the similarity of allele and genotype frequencies.

Results

Throughout this paper the alleles of the SNPs studied are marked with numbers 1 and 2; number 1 denotes alleles that are cut by *ApaI* (VDRA), *BsmI* (VDRB) and *FokI* (VDRF) restriction endonucleases, while number 2 denotes alleles that are uncut by the enzymes.

The genotypes of the patients (all samples + regional) were in Hardy-Weinberg equilibrium for all three SNPs. All controls except those for VDRB from Oulu ($P = 0.014$) were also in Hardy-Weinberg equilibrium.

In case-control association tests we did not detect any statistically significant difference between the groups for VDRA. Borderline significant differences were seen for VDRB, when all controls and all cases, all controls and female patients or all controls and *0302-positive cases were compared ($P = 0.024$, $P = 0.011$ and $P = 0.016$ respectively). Similarly, a significant difference was detected for VDRF when Turku controls and Turku cases were compared ($P = 0.0063$) (Table 2). However, these differences disappeared after correction for multiple testing. The comparisons of data stratified according to the age at diagnosis did not reveal any significant difference between the groups (data not shown).

Allele 2 of VDRF was transmitted to sons in 35.5% (33 transmitted (T), 60 non-transmitted (NT), $P = 0.005$) of HLA DQB1*0302-positive families according to the allele-wise TDT. We found no other statistically significant distortion from the expected 50% transmission for any of the three SNPs studied in any of the stratification groups when all the transmissions and transmissions to sons and to daughters as well as from fathers and from mothers were analysed (Table 3).

When the haplotypes constructed from the three markers were analysed in TDT, no significant distortion was observed when all transmissions were analysed. However, the transmission of the haplotype 2-1-2 (VDRA-VDRB-VDRF) to sons was increased (76 T, 50 NT, T% 60.3, $P = 0.024$), and a decreased (64 T, 99 NT, T% 39.3, $P = 0.0059$) transmission of the haplotype 1-1-2 to sons was observed. The transmission of the haplotype 1-1-2 to sons was also decreased (24 T, 51 NT, T% 32.0, $P = 0.0019$) in families with the HLA DQB1*0302-positive index case. In addition, a reduced (32 T, 53 NT, T% 37.6, $P = 0.022$) transmission of the haplotype 2-2-1 to daughters was detected (Table 4). However, after correction for multiple testing these results did not remain statistically significant. Stratification of the family material according to age at diagnosis did not affect the allele or haplotype TDT results.

Surprisingly, we observed that genotype and allele distributions of VDRA, VDRB and VDRF were different in the three control sets. We detected that Turku (southwest) and Oulu (north) differed from each other significantly at all three sites, while Tampere (central Finland) was in between. The P values obtained from χ^2 tests (genotype-wise) were as follows: Turku versus Oulu: VDRA $P = 0.0002$, VDRB $P = 0.0013$, VDRF $P = 0.018$; Turku versus Tampere: VDRA $P = 0.0065$, VDRB $P = 0.12$, VDRF $P = 0.82$; and Tampere versus Oulu: VDRA $P = 0.12$, VDRB $P = 0.15$, VDRF $P = 0.13$ (Table 2).

Table 2 Genotype frequencies and P values for control subjects and patients with T1D.

	Controls, genotype, %				Cases, genotype, %				P value	
	<i>n</i>	1-1	1-2	2-2	<i>n</i>	1-1	1-2	2-2	Genotypes	Alleles
VDRA										
All	2379	18.5	49.1	32.3	944	18.2	49.8	32.0	0.94	0.98
Turku	797	19.1	55.3	25.6	198	17.7	53.5	28.8	0.65	0.45
Tampere	450	15.3	50.9	33.8	56	23.2	41.1	35.7	0.23	0.86
Oulu	843	19.6	46.1	34.3	239	18.0	48.1	33.9	0.82	0.86
VDRB										
All	2837	45.5	41.7	12.8	1064	42.3	46.4	11.2	0.024	0.51
Turku	844	42.2	46.1	11.7	220	44.1	44.1	11.8	0.92	0.76
Tampere	1175	45.4	41.5	13.1	58	50.0	37.9	12.1	0.79	0.60
Oulu	818	49.3	37.3	13.4	226	40.1	45.6	13.7	0.053	0.087
VDRF										
All	2356	13.2	47.8	39.9	985	14.7	49.0	36.2	0.24	0.1
Turku	808	12.6	51.2	36.1	274	18.2	54.7	27.0	0.0063	0.0027
Tampere	457	13.3	49.5	37.2	55	12.7	50.9	36.4	0.98	0.93
Oulu	795	11.7	45.3	43.0	249	14.9	45.8	39.4	0.34	0.18

Table 3 Allelic TDT results of VDRA, VDRB and VDRF.

	Allele	T	NT	T%	P
VDRA					
All	1	242	255	48.7	0.55
To sons	1	117	145	44.7	0.084
To daughters	1	125	110	53.2	0.33
From fathers	1	113	135	45.6	0.16
From mothers	1	130	121	51.8	0.57
VDRB					
All	1	190	178	51.6	0.53
To sons	1	99	103	49.0	0.78
To daughters	1	91	75	54.8	0.21
From fathers	1	92	88	51.1	0.76
From mothers	1	99	91	52.1	0.56
VDRF					
All	2	234	231	50.3	0.89
To sons	2	122	134	47.7	0.45
To daughters	2	112	97	53.6	0.30
From fathers	2	123	118	51.0	0.75
From mothers	2	112	114	49.6	0.89

Table 4 Results of VDRA-VDRB-VDRF haplotype TDT.

Transmission	Haplotype	T	NT	T%	P
All	1-1-2	139	163	46.0	0.17
	2-2-2	129	116	52.7	0.42
	1-1-1	119	101	54.1	0.22
	2-1-2	122	96	56.0	0.087
	2-2-1	77	103	42.8	0.055
To sons	2-1-1	46	52	46.9	0.59
	1-1-2	64	99	39.3	0.0059
	2-2-2	70	68	50.7	0.89
	1-1-1	71	53	57.3	0.10
	2-1-2	76	50	60.3	0.024
To daughters	2-2-1	45	50	47.4	0.63
	2-1-1	27	32	45.8	0.56
	1-1-2	75	64	54.0	0.35
	2-2-2	59	48	55.1	0.29
	1-1-1	48	48	50	1.0
From fathers	2-1-2	46	46	50	1.0
	2-2-1	32	53	37.6	0.022
	2-1-1	19	20	48.7	0.88
	1-1-2	70	86	44.9	0.19
	2-2-2	64	47	57.7	0.10
From mothers	1-1-1	62	52	54.4	0.34
	2-1-2	59	48	55.1	0.30
	2-2-1	39	57	40.6	0.071
	2-1-1	27	31	46.6	0.61
	1-1-2	69	77	47.3	0.53
From mothers	2-2-2	65	69	48.5	0.70
	1-1-1	57	49	53.7	0.45
	2-1-2	63	48	56.8	0.16
	2-2-1	38	46	45.2	0.38
	2-1-1	19	21	47.5	0.82

Discussion

Several studies have recently reported mostly weak associations of various VDR SNP alleles or haplotypes with T1D (24–31). The results have been ambiguous, and disease association of several different alleles has been shown in a variety of study populations. In order to clarify the contribution of VDR polymorphisms

to genetic susceptibility to T1D among the Finnish patients we analysed an extensive series of controls and cases including 544 nuclear families, the largest material studied to date.

All previously reported associations of VDR SNPs with T1D are derived from rather small series of study subjects. This study had 80% power to detect a difference of approximately 5% in VDRA, VDRB and VDRF allele frequencies between the controls and the patients. The differences in genotype frequencies observed between patients and controls in this study were small and statistical significances disappeared after correction for multiple testing, if the number of different stratification comparisons is used as a correction factor (regions, sex, HLA genotypes, age at onset, alleles/genotypes). However, if only correction for the number of markers studied is made, the VDRF result in the Turku set remains weakly significant. This disease association of the VDRF was not detected in the family material, which confirms the negative finding in the combined case-control comparison. It is well known that correction for multiple testing is a conservative way of interpreting the results and real but weak associations might be lost. However, when a substantial number of comparisons are performed, which is the case in most disease association studies, correction for multiple testing is the only way to minimise the risk of false positive results (type I statistical error) and should therefore be applied (33).

The discrepancy between the previously reported, mainly weakly positive results and our negative results may arise from a series of reasons. First, we noticed an SNP at position 76945 in the GenBank contig NT_009526. This SNP is located in the primer region for the BsmI PCR-product used in several previous studies (24, 26, 29, 31). By using a primer three nucleotides shorter than that previously utilised we detected a preferential amplification of a DNA strand with a complementary SNP allele resulting in the misinterpretation of certain BsmI heterozygous genotypes as homozygous ones. This might have remained undetected without studying families. In our series, the frequency of heterozygous VDRB samples was higher than 40%, whereas it was reported to be very low in Taiwanese subjects (patients 11.0%, controls 6.5%) (26). This might indicate that the above-mentioned SNP was overlooked. A similar bias could have affected the results of other studies reporting BsmI frequencies based on the use of the same primer (24, 29, 31). Secondly, it is believed that T1D has several different aetiological pathways. The populations might, therefore, differ from each other also in VDR-related susceptibility to T1D. Thirdly, the previous studies have been based on rather small patient series. This could lead to unintended deviations, when the study subject series are compiled. Fourthly, we interpreted the results after correction for multiple testing, which was not done in all previous surveys. Finally, if the VDR itself is not

regarded as a disease affecting locus, but rather a marker locus in linkage disequilibrium with the real disease locus, the divergent findings might originate from variable strength of linkage disequilibrium in different study populations (34).

Finland is usually regarded as a genetically very homogenous country (35) and sample collection for large case-control studies is believed not to be confounded by population stratification. However, we found statistically significant differences in the background frequencies of all three SNP genotypes between the Turku, Oulu and Tampere series. Case-control comparisons were carried out separately for the samples from the different centres and also for the total study cohort, since the Turku and Oulu sample series were similar in size and could therefore be combined, even though there were differences in allele frequencies. In the transmission disequilibrium analyses the confounding effect of population stratification is not an issue, since it is based on transmissions within families, not between population samples.

Turku and Oulu are located approximately 650 km from each other, Turku on the southwest coastline of Finland, whereas Oulu is situated in northern Finland. Due to the more northern location, the population in the Oulu region experiences less yearly solar energy. More than 90% of vitamin D in the peripheral circulation is produced endogenously in the skin by exposure to ultraviolet radiation. It has been shown that uncut alleles of the VDRA and VDRB polymorphisms increase receptor function and upregulate vitamin D-induced protein expression (36). In this study we have shown that the background population in the Oulu region has higher frequencies of the uncut VDRA and VDRB genotypes when compared with the population in the Turku area. Considering the results of Morrison *et al.* (36) and the data on yearly solar energy, it might be speculated that people living in northern latitudes and thus obtaining less solar energy for vitamin D production in the skin need 'more effective' alleles of the VDR variants. It is possible that due to the variation in the ultraviolet radiation a considerable difference in the background frequencies of VDR polymorphisms exist across Europe and this could have an effect on the findings of disease association studies. The lack of Hardy-Weinberg equilibrium in the Oulu controls for VDRB is caused most probably by sampling factors, since all other sample sets, including Oulu patients, were observed to be in equilibrium.

In conclusion, we combined case-control and affected-family approaches for the analysis of a large representative series of patients and controls to detect the possible association between VDR SNPs and T1D in Finland. Some marginal differences were observed, but after correction for multiple testing all those disappeared. We suggest that the three SNPs of the VDR gene studied are not associated with T1D in the Finnish population.

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