VDR polymorphisms influence the immune response in type 1 diabetic children from Santiago, Chile

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Abstract

Objective: To evaluate the influence of ApaI, BsmI and TaqI polymorphisms of the VDR gene and HLA-DQB1* alleles in type 1 diabetic children and to assess their possible relationship with circulating levels of 25-hydroxyvitamin D_3, auto-antibodies, and INFγ/TGFβ1 cytokines levels in Chilean cases and controls.

Methods: DNA and serum samples from 216 newly diagnosed type 1 diabetic and 203 unrelated control children were evaluated for IA-2 and GAD_{65} auto-antibodies, 25-hydroxyvitamin D_3 levels, HLA-DQB1* alleles, and VDR gene polymorphisms.

Results: The frequency of the b allele and the bb genotype in type 1 diabetic patients was significantly lower compared with the control group (0.635 versus 0.749, $p < 0.01$ and 0.370 versus 0.567, $p < 0.04$). 25-Hydroxyvitamin D_3 levels showed no differences between type 1 diabetic and healthy children. In cases, 25-hydroxyvitamin D_3 levels were not associated with a special auto-antibodies profile according to the presence or absence of $GAD_{65}{}^+$ or IA-2^+. The haplotype combination BAT was higher in cases (0.062 versus 0.019, $p < 0.0022$) and bAT was more frequent in controls (0.266 versus 0.180, $p < 0.003$). In cases, the aaBbTT genotype showed the most significant increase in TGFβ1 level across the VDR categories. Finally, when considering the HLA class II risk genotype (DQB1*0302) and the VDR genotypes (AabbTT and aabbTT), higher levels of GAD_{65}, IA-2 and TGFβ1 were observed among diabetic children.

Conclusion: We found an association between a VDR polymorphism (BsmI) and type 1 diabetes. An association was found of AabbTT and aabbTT genotypes and the HLA-DQB1*0302 allele with high levels of GAD_{65}, IA-2 and TGFβ1.

Keywords: Type 1 diabetes; VDR polymorphism; Vitamin D; Auto-antibodies

1. Introduction

Type 1 diabetes mellitus (DM1) is a complex disease characterized by the autoimmune and progressive destruction of insulin-secreting pancreatic β-cells. Both genetic and environmental factors are generally accepted as main participants in this autoimmune process that leads to the onset of the disorder [1,2]. For more than 20 years, the most firmly established known genetic contribution to DM1 susceptibility has been the human leukocyte antigen region (HLA) on the short arm of chromosome 6 [1,3,4], and it has been the focus of a great number of association studies worldwide [5–7].

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However, several other non-MHC regions have been identified as predisposition factors [8–11]. During the last years, diverse studies have emerged to analyze a possible relationship between DM1 and polymorphisms in the Vitamin D receptor (VDR) gene region on chromosome 12 [12–17].

The biological actions of 1,25-$(OH)_2D_3$ are mediated through the VDR [18,19], a nuclear receptor that belongs to the superfamily of ligand activated transcription factors [20,21]. It is not surprising that Vitamin D exerts a multitude of biological functions beyond calcium and phosphorus metabolism [22–24]. Common autoimmune diseases like rheumatoid arthritis, multiple sclerosis, lupus and type 1 diabetes have all been successfully prevented in animal models that received 1,25-$(OH)_2D_3$ [25–29]. Finally, population studies in humans have reported results indicating that Vitamin D supplementation in early childhood effectively decreases DM1 incidence [30,31]. On the other hand, 1α,25-dihydroxyvitamin D_3 inhibits T-cell proliferation and eventually decreases the production of Th1 cytokines (IL-2, INFγ) and also activates TGFβ1 and IL-4 expression [19]. All of this is achieved by inhibiting Th1 lymphocyte subset differentiation and by enhancing the reactivity of regulatory T-cells [23]. Vitamin D is involved in the differentiation and maturation of dendritic cells, critical in immune response induction [28].

The aim of this work was to evaluate the case–control differences in three common polymorphisms of the VDR gene in type 1 diabetic and healthy children from Santiago, Chile, and to study their influence on DM1 susceptibility and their possible relationship with HLA class II alleles (DQB1*0302/*0201), GAD_{65} and IA-2 auto-antibodies, circulating levels of 25-hydroxyvitamin D_3 and cytokines TGFβ1–INFγ, in order to evaluate if there is any association with immunosuppressive functions.

2. Subjects and methods

2.1. Subjects

The study was carried out in Santiago, Chile, with incident cases of type 1 diabetes diagnosed by means of standardized methods (WHO DiaMond Project) during the years 2004–2006. A total of 216 new cases (mean age 9.3 ± 4.2 years, 120 boys and 96 girls) were compared to 203 healthy unrelated control children (mean age 10.3 ± 2.5 years, 106 boys and 97 girls). All blood samples among the type 1 diabetic patients were obtained in a short period of time near to diagnosis (0–3 weeks). The control group represented volunteers who participated from eight schools in Santiago, with similar characteristics of Hispanic surnames and socioeconomic conditions. The Chilean population is a melt of people who came from different parts of the world, including different European countries (mostly from Spain), and Amerindians [32]. Previous studies have defined three socio-genetic strata in the population of Santiago, Strata I (high income, pure Caucasian, represents 7% of the population); Strata II (medium income, mixed population, represents 65% of the population) and Strata III (low income, high percentage of aboriginal ancestry, 18% of the population). The sample for this study is mainly representative of Strata II and III [33]. The incidence of the disease in the metropolitan region of Santiago, the main nucleus of urban population in Chile, has shown a strong increase in the last 18 years, from 2.5 cases per 100,000 inhabitants in 1986, to 7.5 per 100,000 inhabitants in the 2003 period [34].

Information regarding allergies, food intolerance, familial history of diabetes and other autoimmune diseases was collected from the children's mothers by means of a questionnaire. All patients and controls or their parents gave informed consent and this study was approved by the Local Ethical Committee.

2.2. DNA isolation and VDR genotyping

From every patient and control we extracted and obtained 3 ml of peripheral blood. From each sample, serum supernatant and solid residues were separated by centrifugation. A total of 500 μl of serum was saved for auto-antibodies and cytokines analysis. Genomic DNA was extracted from peripheral blood using a standard protocol (Winkler, Santiago, Chile). Genotypes for the polymorphic restriction sites of the VDR gene were obtained by DNA amplifications with standard PCR and specific sets of primers, followed by the restriction fragment length polymorphism method (RFLP). We used ApaI, BsmI and TaqI restriction enzymes (New England Biolabs, UK). We performed one polymerase chain amplification with two different sets of primers: forward primer 5-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3 and reverse primer 5-AAC CAG CGG GAA GAG GTC AAG GG-3 for BsmI, and forward primer 5-CAG AGC ATG GAC AGG GAG CAA-3 and reverse primer 5-GCA ACT CCT CAT GGC TGA GGT CTC-3 for ApaI and TaqI. The PCR conditions for the three polymorphisms were 94 °C for 4 min and 30 cycles with the following characteristics: 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, with a final extension step at 72 °C for 5 min. At the end of each PCR, we performed a checkpoint for amplification on a 1% agarose gel. The gels were visualized by ethidium bromide staining under ultraviolet light. Digestions for BsmI and TaqI were performed with 2 U enzyme and 8 μl PCR product at 65 °C for 2 h, and digestion for ApaI was performed under the same conditions but with a 37 °C restriction temperature. The results of the RFLPs were viewed in a 2% agarose gel.

2.3. HLA DQB1*0302 and 0201 alleles determination

The HLA-typing for class II alleles was performed by the routine test. The HLA-DQB1 alleles were typed using the

InnoLiPa reverse slot blot hybridization system provided by Murex (Immunogenetics, Zwijndzecht, Belgium).

2.4. 25-Hydroxyvitamin D_3 detection, auto-antibody measurement and cytokines levels

Serum concentrations of 25-hydroxyvitamin D_3 were determined in triplicate using a Radioimmunoassay from DiaSorin (Stillwater, MN, USA). These assays have been well characterized and recognize both the D_2 and D_3 forms of the vitamin (inter-assay coefficient: 3.1%; intra-assay coefficient: 5.3%). The selection of 25-hydroxyvitamin D rather than 1,25-$(OH)_2$ Vitamin D was made on the basis of the half-life of this metabolite, to avoid the differences between children recruited at debut and during the first 3 weeks after debut. Screening for serological anti-GAD_{65} and anti-IA-2 auto-antibodies was performed by Radioimmunoassay (^{125}I-RIA kit) from DRG Diagnostic (Mountainside, NJ, USA). For anti-GAD_{65} and anti-IA-2, we assigned positives when samples showed concentrations over 0.9 and 0.75 U/ml, respectively, according to manufacturer's recommendations. In both determinations the functional assay sensitivity generally represents that concentration which corresponds to the 10% (intra-assay) and to the 20% (inter-assay) coefficient of variation in the respective precision profiles of the assay in the lower concentration range. Upon correct and thorough performance of anti-GAD_{65} and anti-IA-2 RIA, this value was found at approximately 0.6 and 0.7 U/ml, respectively. Cytokines INFγ and TGFβ1 were measured by an enzyme-linked immunosorbent assay (ELISA) development system (R&D system, Oxon, UK), with an inter-assay coefficient of 4.1% and an intra-assay coefficient of 3.3%.

2.5. Statistical analyses

Comparisons of the haplotype or genotype frequencies between cases and controls were performed using the chi-square test or the Fisher's exact test using the online SHEsis package (URL: http://202.120.7.14/analysis/myAnalysis.php). Comparisons of continuous variables across study groups were performed with the Student's *t*-test, two-sample Wilcoxon rank sum (Mann–Whitney test). The distribution of cytokines among composed genotypes HLA and VDR were analyzed by means of Kruskal–Wallis test. Also, a Hardy–Weinberg test was performed for each polymorphisms including in this research. A *p*-value of less than 0.05 was considered statistically significant.

Table 1
Distribution of VDR gene polymorphisms in patients with type 1 diabetes and non-diabetic control children from Santiago, Chile

VDR polymorphism	Cases ($n = 216$)		Controls ($n = 203$)	
	n	Frequency	*n*	Frequency
BsmI				
BB	21	0.101	14	0.069
Bb	110	0.529	74	0.365
bb	77	0.370*	115	0.567
TaqI				
TT	115	0.532	121	0.596
Tt	79	0.366	69	0.340
tt	22	0.102	13	0.064
ApaI				
AA	54	0.254	43	0.212
Aa	115	0.540	125	0.616
aa	44	0.206	35	0.172

* $p < 0.04$ (comparison between cases and controls).

2.6. Results

2.6.1. VDR alleles and genotypes

Table 1 shows the VDR polymorphisms distribution observed in cases and healthy children. The bb genotype frequency was significantly lower in cases than in controls (0.370 in cases; 0.567 in controls, $p < 0.04$). The allelic frequency of "*b*" was higher in controls compared with cases (74.9% versus 63.5%, $p < 0.01$). No statistically differences were observed for "*t*" allelic frequency 28.5% in cases and 23.4% in controls ($p = \text{NS}$) and for "*a*" allelic frequency 47.7% in cases and 48.0% in controls ($p = \text{NS}$).

Table 2 shows the complex VDR haplotypes in cases and controls. We detected five prevalent haplotypes in both groups that represent more than 95% of the haplotypes, where BAT was more frequent in cases (0.062 versus 0.019, OR = 3.32, $p < 0.0022$) and bAT was more frequent in controls (0.266 versus 0.180, OR = 0.66, $p < 0.003$). Among the genotype combinations, AabbTT was higher in controls compared to

Table 2
VDR haplotypes distribution among the Chilean population

VDR haplotype	Cases ($n = 216$)		Controls ($n = 203$)		OR IC95%	*p*
	n	Frequency	*n*	Frequency		
BAT	25	0.062	8	0.019	3.32 (1.47–7.46)	0.0022
bAT	74	0.180	108	0.266	0.66 (0.43–0.84)	0.003
BaT	13	0.032	6	0.016	2.08 (0.80–5.45)	0.124
baT	182	0.443	189	0.465	0.92 (0.69–1.21)	0.531
BAt	111	0.270	88	0.216	1.34 (0.97–1.85)	0.075

All those frequencies <0.03 were ignored in the analysis.

Table 3
Main VDR genotypes and serum 25-hydroxyvitamin D, INFγ, TGFβ1 levels in Chilean type 1 diabetic children and controls

	25-Hydroxyvitamin D (ng/ml)	INFγ (pg/ml)	TGFβ1 (pg/ml)
Cases ($n = 208$)			
AABBtt ($n = 20$)	25.2 ± 10.8	94.1 ± 61.3	330.1 (192.9–1827.1)
AABbTt ($n = 19$)	26.4 ± 10.7	89.6 ± 45.6	267.6 (131.2–1578.6)
AABbTT ($n = 7$)	35.1 ± 18.6	128.4 ± 84.8	307.5 (254.7–1198.6)
AAbbTT ($n = 4$)	26.3 ± 1.5	83.1 ± 12.2	142.3 (25.1–152.6)
AaBbTT ($n = 16$)	21.7 ± 4.4	82.3 ± 62.3	497.4 (167.3–1921.4)
AaBbTt ($n = 61$)	29.9 ± 1.2	60.8 ± 52.8	318.8 (147.8–2164.3)
AabbTT ($n = 38$)	25.5 ± 4.6	81.1 ± 23.9	342.2 (156.7–2945.7)
aaBbTT ($n = 9$)	26.7 ± 11.7	86.9 ± 15.6	1332.2 (214.1–2800.1)*
aabbTT ($n = 34$)	25.0 ± 7.5	71.0 ± 66.2	305.2 (153.8–2648.6)
Controls ($n = 203$)			
AABBtt ($n = 12$)	26.2 ± 7.7	92.1 ± 9.7	269.8 (164.3–431.1)
AABbTt ($n = 18$)	25.3 ± 6.8	68.4 ± 32.7	241.2 (167.3–370.8)
AABbTT ($n = 8$)	27.8 ± 4.5	81.3 ± 13.3	188.3 (144.7–350.8)
AAbbTT ($n = 18$)	23.7 ± 12.0	97.8 ± 17.3	358.8 (280.4–388.9)
AaBbTT ($n = 7$)	26.1 ± 11.5	100.3 ± 31.1	358.8 (280.4–388.9)
AaBbTt ($n = 42$)	28.1 ± 7.9	92.1 ± 10.8	235.2 (135.7–369.3)
AabbTT ($n = 60$)	27.2 ± 7.1	91.0 ± 12.2	233.6 (146.2–506.4)
aaBbTT ($n = 4$)	24.7 ± 3.2	101.2 ± 11.6	279.6 (212.6–477.8)
aabbTT ($n = 43$)	25.8 ± 7.5	91.3 ± 11.5	213.3 (144.7–397.9)

Values are expressed as mean ± S.D. or median and range.
* $p < 0.025$.

diabetic cases (30.5% versus 18.7%, $P_c = 0.035$). According to the Hardy–Weinberg equilibrium test, almost all the genotypes distributions tested were not statistically different from the distribution predicted by this analysis. That was the case for the TaqI (p-value 0.4603) and BsmI (p-value 0.657) polymorphisms. However, ApaI showed significant different in cases (p-value 0.0008).

2.6.2. Auto-antibodies GAD_{65} and IA-2

Positive values for auto-antibodies were found in both study groups: in cases ($n = 216$), 59.2% of positive title for anti-GAD_{65} (1.5% in controls) and 51.4% of positive title for anti-IA-2 (3.2% in controls). No preferential relationship was observed between the distribution of the positive auto-antibody profile and the VDR genotypes.

2.6.3. VDR genotype, 25-hydroxyvitamin D levels, INFγ and TGFβ1 serum concentrations

In relation to serum 25-hydroxyvitamin D_3 concentrations, in overall group no differences were observed between type 1 diabetic patients (26.8 ± 7.7 ng/ml) and healthy children (28.8 ± 4.1 ng/ml). Serum 25-hydroxyvitamin D_3 levels only were different in cases according to the age of diagnosis: 0–4 years (23.9 ± 7.7 ng/ml); 5–9 years (25.9 ± 8.7 ng/ml); 10–14 years (29.6 ± 6.7 ng/ml) ($p < 0.033$). No differences were observed in overall group for INFγ levels between cases and controls (87.6 [9.3–228.8] pg/ml versus 88.3 [9.5–178.5] pg/ml). TGFβ1 concentrations showed a significant difference when we compared cases and controls (318.8 [126.7–2945.7] pg/ml versus 237.4 [90.1–506.4] pg/ml, $p < 0.02$).

In addition, we analyzed the distribution of serum 25-hydroxyvitamin D_3 levels and INFγ and TGFβ1 concentrations in each VDR genotype in cases and controls. Table 3 show that the VDR genotype AaBbTT has a low concentration of 25-hydroxyvitamin D in cases compared with all the other genotypes. On the other hand, a significantly high level of TGFβ1 was observed in the VDR genotype aaBbTT ($p < 0.025$ compared with all values in cases and controls).

Finally, Table 4 shows the results among type 1 diabetics when we stratified the cases according to HLA DQB1 markers and VDR genotypes. An association was observed of the HLA^2 genotype (presence of the DQB1*0302 allele) and VDR genotypes AabbTT or aabbTT with a high positive title of GAD_{65} and IA-2 auto-antibodies (83% and 81%, respectively) and high levels of TGFβ1 (502.6 [153.6–2945.7] pg/ml) ($p < 0.02$) compared with the others genotypes combinations.

3. Discussion

The molecular mechanisms underlying type 1 diabetes are only partly understood. It develops as a result of a complex interaction of many genetic and environmental factors leading to the immune destruction of the insulin-producing β-cells [1].

During the last decade, several VDR gene polymorphisms have been shown to be associated with autoimmune diseases [14–17]. The alleles or genotypes associations in VDR are contradictory in the literature. While in type 1 diabetic patients from Taiwan, it has

Table 4
Serum immune markers according to HLA-DQB1* and VDR genotype in cases

	Cases		GAD_{65}+	IA-2+	TGFβ1 (pg/ml)	INFγ (pg/ml)
	n	Frequency				
HLA^2 and VDR^2	48	0.235	83%	81%	502.6* (153.6–2945.7)	70.4 (11.0–167.5)
HLA^2 and VDR^1	53	0.261	67%	55%	345.0 (131.2–2164.3)	88.9 (15.4–175.4)
HLA^2 and VDR^0	32	0.160	50%	61%	308.3 (126.7–1486.9)	120.1 (41.2–173.1)
HLA^1 and VDR^2	19	0.095	58%	40%	346.2 (131.2–1916.3)	68.4 (12.3–167.5)
HLA^1 and VDR^1	20	0.098	63%	30%	325.0 (167.0–2345.7)	88.9 (9.4–211.3)
HLA^1 and VDR^0	14	0.072	56%	31%	281.3 (129.2–955.4)	94.6 (25.2–228.8)
HLA^0 and VDR^2	5	0.026	47%	63%	271.1 (131.2–2164.3)	66.4 (9.3–167.5)
HLA^0 and VDR^1	7	0.033	58%	50%	256.1 (141.6–2200.5)	98.9 (15.4–177.4)
HLA^0 and VDR^0	4	0.020	51%	52%	234.3 (161.2–1455.1)	92.1 (41.2–173.1)
Total	202	1.00	59.2%	51.4	318.8 (126.7–2945.7)	87.6 (9.3–228.8)

HLA^2: carriers of allele HLA-DQB1*0302 (one or two copies); HLA^1: carriers of allele HLA-DQB1*0201 (one or two copies); HLA^0: absence of alleles HLA-DQB1*0302 and HLA-DQB1*0201; VDR^2: AabbTT or aabbTT genotype; VDR^1: AABbTt or AaBbTt genotype; VDR^0: other VDR combinations.

* $p < 0.02$ Kruskal–Wallis test.

been shown that the disease was shown to be associated with the B allele [15], in Southern Indian families, the preferential transmission of the b allele to affected subjects was observed [12]. Similar results were observed in type 1 diabetic cases from Barcelona [35].

In this study, the distribution of genotypes was not significantly different from the expectation under Hardy–Weinberg equilibrium for TaqI (p-value 0.4603) and BsmI (p-value 0.657) polymorphisms. Though, ApaI was different in cases. There are several possible explanations for the existence of allelic deviation from the Hardy–Weinberg equilibrium. One possibility is that there is population stratification in the samples, which is always a concern in population-based studies. It is best addressed by using family based studies (TDT analysis), such as our previous report in this polymorphism [36]. The other possibility is biased sampling of patients or non-random mating in this population. According to our patient recruitment protocol, there was no evidence of this.

Our study found results similar to those reported by Chang et al. [14] with a small contribution of the VDR gene polymorphism to auto-antibodies (GAD, IA-2 antibodies). A different relationship was found by Motohashi et al. [37] in respect of an association between a VDR gene polymorphism and acute-onset type 1 diabetes, regardless of the presence or absence of islet-associated auto-antibody.

SNPs BsmI, ApaI and TaqI are in linkage disequilibrium, and their haplotypes were previously shown to be associated with type 1 diabetes in the German population [13]. However, recent data in a broad familial study found no evidence of association in the UK [38].

In other studies, different combinations for VDR haplotypes have been proposed [13–15]. The genotype combination which conferred strongest susceptibility to type 1 diabetes in a Dalmatian population from South Croatia was BBAAtt ($p = 0.002$) [39]. Interestingly, the BAt haplotype was found to be a risk factor in a German population [13]. Our results showed a high frequency of this combination in cases (0.270), but without a significant difference with respect to controls (0.216).

On the other hand, certain variants may be only functionally important among subjects with low levels of Vitamin D [40,41]. Nejentsev et al. [38] analyzed the effect of a patient's country of origin and a combined effect of the patient's year of birth and country of origin on the association between type 1 diabetes and eight VDR SNPs in five different populations. They did not find any evidence for significant heterogeneity of the type 1 diabetes association. This information may be consistent with our case–control study, where the origin of the Chilean population should be considered as an admixed group with a high impact of European genes on the native background [33]. A study from several European countries revealed that Vitamin D supplementation in early childhood seems to be associated with a reduction in the incidence of type 1 diabetes [31].

The exact immune effects of Vitamin D administration are controversial; VDRs might modulate the disease course of type 1 diabetes. As was speculated by Motohashi et al. [37], the effects of Vitamin D administration, mediated through the VDR, may be affected by VDR gene polymorphisms. Our results showed a relationship between the low levels of 25 OH Vitamin D and recently diagnosed cases in the age range

of 0–4 years, probably in concordance with the aggressive presentation of the disease, but without association with a special distribution of VDR genotypes. On the other hand, according to the recent consensus for 25 OH Vitamin D [40], no differences were detected between patients and healthy children in the percentages of deficiency or insufficiency of 25 OH Vitamin D_3 or normal concentrations in our country.

The presence of the VDR in peripheral blood monocytes and activated T cells has suggested a possible relationship between Vitamin D and the immune system. Vitamin D induces an autoantigen-specific ''protective'' Th2 cell population, not only at the site of the β cell attack but also in the peripheral immune system [42]. Vitamin D also has a direct effect on naïve CD4+ T cells to enhance the development of Th2 cells in the absence of APC [43]. Our data showed a possible interaction effect between the HLA-DQB1*0302 allele and some specific VDR genotypes that increased the immune response mediated by TGFβ1.

Several studies have shown evidence for the putative role of VDR polymorphism in the etiology of type 1 diabetes as a controversial point [41]. The current debate on VDR and Vitamin D levels in type 1 diabetes [40] should be analyzed by means of intervention trial during the first months of life, to establish the possible consequences of supplementation with Vitamin D in individuals genetically at risk (VDR and other candidate genes) for type 1 diabetes with antecedents of altered immunological response.

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