Lack of association of Vitamin D Binding Protein Polymorphisms in Egyptian Children with Type 1 Diabetes

Mısırlı Çocuklarda Tip 1 Diyabet ile D Vitamini Bağlayıcı Protein Polimorfizmi Arasındaki İlişki Yokluğu

Moushira Mahmoud, Gehan Ibrahima, Manal Fawzya, Hoda Atwa*

Faculty of Medicine, Suez Canal University, Department of Medical Biochemistry, Ismailia, Egypt
*Faculty of Medicine, Suez Canal University, Department of Pediatrics, Ismailia, Egypt

Abstract

Background: Type 1 diabetes mellitus (T1D) is a polygenic autoimmune disease. Vitamin D is a potent modulator of the immune system and has been implicated in T1D pathogenesis and prevention. Vitamin D-binding protein (VDBP) is the main systemic transporter of vitamin D and is essential for its cellular endocytosis. Two frequent single nucleotide polymorphisms (SNPs), in exon 11 of the VDBP gene, result in amino acid variants: (rs7041) GAT→GAG substitution replaces aspartic acid by glutamic acid in codon 416; and (rs4588) ACG→AAG substitution in codon 420 leads to an exchange of threonine for lysine. These VDBP variants lead to differences in the affinity for vitamin D.

Objective: The objective was to evaluate the association of these 2 VDBP gene SNPs with T1D in Egyptian children.

Material and Method: Unrelated 59 children with T1D and 65 healthy controls were included in this study. The sequence of VDBP exon 11, which contains both examined SNPs, was analyzed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Results: The frequency of Asp/Glu alleles, at codon 416, was 35.6/64.4% in T1D patients and 44.6/55.4% in controls (P=0.15). At codon 420 the frequency of Thr/Lys alleles were 88.1/11.9% and 87.7/12.3% (P=0.91), respectively. Distributions of genotypes at both loci, and the common haplotypes constructed by them, were also very similar in both groups (P> 0.05).

Conclusion: DNA polymorphisms in the VDBP gene are not associated with T1D in Egyptian children.. *Turk Jem 2011; 15: 111-5*

Key words: Type 1 diabetes, Vitamin D binding protein, group-specific component, Asp416Glu, Thr420Lys

Özet

Giriş: Tip 1 diyabet (T1D), bir poligenik otoimmün hastalıktır. D vitamini, immün sistemin güçlü bir düzenleyicisidir ve T1D patogenezi ve önlenmesi ile ilişkilendirilmiştir. Vitamin D bağlayıcı protein (VDBP), D vitaminin temel sistemik taşıyıcısıdır ve hücresel endositoz için gereklidir. VDBP geninde exon 11'de sık görülen iki tek nükleotid polimorfizmi (SNP), amino asit varyantlarının oluşmasına yol açar: (rs7041) GAT → GAG değişimi sonucu kodon 416'da glutamik asit yerine aspartik asit geçer; ve kodon 420'de (rs4588) ACG → AAG değişimi ile lizin yerine treoninin geçer. Bu VDBP varyantları, D vitaminine olan affinitede farklılıklara yol açar.

Amaç: Bu çalışmada Mısırlı çocuklarda, bu iki VDBP geni SNP'lerinin T1D ile ilişkisinin araştırılması amaçlandı.

Metod: Akrabalık bağı olmayan 59 T1D'li çocuk ve 65 sağlıklı kontrol çalışmaya dahil edildi. Araştırılan her iki SNP'yi içeren VDBP exon 11 sekansı, polimeraz zincir reaksiyonu- restriksiyon parçacık uzunluk polimorfizmi (PCR-RFLP) yöntemiyle incelendi.

Bulgular: Kodon 416'da Asp/Glu allel sıklığı T1D hastalarında % 35.6 / 64.4 ve kontrollerde % 44.6 /55.4 şeklindeydi (P=0.15). Kodon 420'de Thr/Lys allel sıklığı ise sırasıyla % 88.1/ 11.9 ve % 87.7 / 12.3 tü (P=0.91). Her iki lokustaki genotiplerin ve bunların oluşturduğu ortak haplotiplerin dağılımı her iki grupta benzer bulundu (P > 0.05).

Sonuç: Mısırlı çocuklarda, VDBP genindeki DNA polimorfizmleri, T1D ile ilişkili bulunmamıştır. *Türk Jem 2011; 15: 111-5*

Anahtar kelimeler: Tip 1 diyabet, Vitamin D bağlayıcı protein, grup-spasifik bileşen, Asp416Glu, Thr420Lys

Address for Correspondence: Moushira Mahmoud MD, Medical Biochemistry Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt 41522
E-mail: moushira@yahoo.com **Recevied:** 05.06.2011 **Accepted:** 14.07.2011

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Introduction

Type 1 (insulin-dependent) diabetes mellitus (T1D) is an autoimmune disease characterized by selective destruction of insulin-secreting beta cells in the pancreatic islets (1). Beta-cell destruction is preceded by insulitis, a massive invasion of the islets by a mixed population of lymphocytes and macrophages; in which T helper 1 (Th1) cytokines produced by islet-infiltrating leukocytes dominate over T helper 2 cells (Th2) (2). 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. Previous studies support a role for vitamin D in the pathogenesis of T1D. 1α, 25-dihydroxyvitamin D3 [1, 25(OH) 2D3], the active form of vitamin D, has been successfully used to prevent autoimmune insulitis and reduce diabetes incidence in the mouse model of T1D (3). In humans, population studies suggest that vitamin D supplementation in early childhood decreases T1D incidence (4). Moreover, the Diabetes Autoimmunity Study in the Young (DAISY) revealed that dietary vitamin D intake by women during pregnancy was correlated with diminished islet autoantibodies in their children (5)

The major biological effect of vitamin D is the regulation of serum calcium and phosphate homeostasis as well as bone metabolism. However, its role in T1D is thought to be related to immunomodulatory actions. Vitamin D induces an autoantigen-specific 'protective' Th2 cell population, blocks pancreatic infiltration of Th1 cells and inhibits their cytokine secretion (6-8).

Thus, it is feasible that genes encoding proteins participating in vitamin D metabolism may influence susceptibility to T1D. A key protein in vitamin D metabolism is the vitamin D binding protein (VDBP), formerly known as "group-specific component" (Gc). VDBP binds to vitamin D metabolites (e.g., 25-hydroxyvitamin D3 [25(OH) D3], the major circulating metabolite, and 1,25(OH)2D3) at the sterol binding domain. It transports vitamin D to liver, kidney, bone, and other target tissues; and stores and prolongs the half-life of the circulating vitamin D metabolites (9, 10). VDBP, a 458-amino acid highly polymorphic single-chain serum glycoprotein, is synthesized and secreted by the liver. The human VDBP-gene is localized at chromosome 4q11–q13. It extends over 35 kb DNA and contains 13 exons and 12 introns (11, 12).

There are three major electrophoretic variants of the VDBP glycoprotein, which differ in their affinity for active vitamin D. These variants are identified by two missense polymorphisms in exon 11: (rs7041) a GAT to GAG substitution at codon 416 that replaces aspartic acid by glutamic acid (Asp416Glu) and (rs4588) an ACG to AAG substitution at codon 420 that changes threonine to lysine (Thr420Lys). Haplotypes of these nucleotide changes result in the variants of the VDBP protein which called Gc1 fast (Gc1F), Gc1 slow (Gc1S) and Gc2 (13, 14).

Few published studies, evaluating the role of VDBP exon 11 polymorphisms in the susceptibility to T1D, gave inconsistent results (15-17). The aim of the present study was to examine the role of VDBP amino acid variants at codons 416 and 420 in the genetic susceptibility to T1D in an Egyptian population.

Materials and Methods

Subjects

Fifty-nine T1D children (median age: 13 years; 27 boys and 32 girls), followed-up in the Pediatric Diabetology Clinic at the Suez Canal University Hospital, were enrolled. The age at the onset of diabetes varied between 2 and 17 years (median: 7.5 years). The mean duration of diabetes was 4.7±2.7 years and the mean value of HbA1c was 9.1±1.9%. T1D was diagnosed according to the criteria specified in the 1999 World Health Organization report (18).No other autoimmune or endocrine diseases were present at the time of the blood collection. The control group consisted of 65 unrelated healthy blood donors (31 males and 34 females; matched for gender to the patients group) who had no family history of diabetes or autoimmune diseases and from the same region as the patients. They were older than 24 years, as recommended by the DiaMond protocol (19). The study was carried out in accordance with the guidelines of the Helsinki Declaration. Informed consents were obtained from parents of all patients and controls.

Genotyping

Genomic DNA was extracted from whole blood using the Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. The concentration and purity of extracted DNA was measured using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). DNA was stored at - 20°C. until ready for use.

The VDBP polymorphisms in exon 11 [Asp416Glu and Thr420Lys] were determined by polymerase chain reactions - restriction fragment length polymorphism (PCR-RFLP). The PCR were carried out in a total volume of 30 µl with 350 ng of genomic DNA, 2.5 U Taq polymerase in 1X Taq polymerase buffer (Promega, Madison WI), 1.5 mmol MgCl2, 0.2 mM of each dNTP and 100 pmol of each primer (Forward: 5'-CAAGTCTTATCACCATCCTG-3' and reverse: 5'-GCCAAGTTACAATAACAC-3') (17). PCR amplifications were performed using Robocycler® gradient 96 thermocycler (Stratagene, USA) according to the following cycling conditions: initial denaturation at 95°C for 10 min, followed by 35 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 10 min. Amplified products (809 bp) were checked by electrophoresis in 2% agarose gel stained with ethidium bromide and compared with the 100-bp DNA size marker (Bioron, Germany).

Then, 10 µl of the amplified products were digested, at 37°C overnight, in a 20 µl reaction containing either 2.5U HaeIII or 2.5U StyI restriction enzymes (Fermentas, Germany) for both Asp416Glu and Thr420Lys polymorphisms, respectively. The digestion products were visualized on 2% agarose gel stained with ethidium bromide. In VDBP Asp416Glu polymorphism, individuals homozygous for the Glu allele showed two bands (577 and 232 bp) while individuals homozygous for the Asp allele had a non-digested band at 809 bp (Figure 1A). The homozygous Thr allele of

the Thr420Lys polymorphism appeared as a single band (809 bp), while, the Lys allele showed two bands (584 and 225 bp) (Figure 1B). The Gc haplotypes were determined by observing the digestion products of both restriction enzymes. The Gc1F has neither HaeIII nor StyI site. The Gc1S has the HaeIII but not the StyI site. The Gc2 has the StyI but not the HaeIII site. The existence of both restriction sites on a single haplotype has not yet been described (20).

Data Analysis

The genotypes and alleles frequencies of the studied polymorphisms were calculated by counting. Hardy–Weinberg equilibriums were assessed on a contingency table of observed and expected genotype frequencies using the Chi-square (χ 2) goodness of fit test. Differences in genotype, allele and haplotype frequencies were calculated by χ 2, or Fisher's exact test when necessary. Two-tailed p-values were calculated and statistical significance was set at $p<0.05$. Odds ratios (OR) and the corresponding 95% confidence intervals (95% CI) were calculated for each genotype, allele, and haplotype. Analysis of data was carried out using SPSS package version 10.0 for Windows (Chicago, Illions, USA).

Results

The frequencies of the Asp416Glu and Thr420Lys polymorphisms in exon 11 of the VDBP gene were examined in T1D patients and controls. The genotype distribution of both studied groups fits the Hardy-Weinberg equilibrium (P> 0.05, Table 1). Both polymorphisms showed no statistically significant difference in

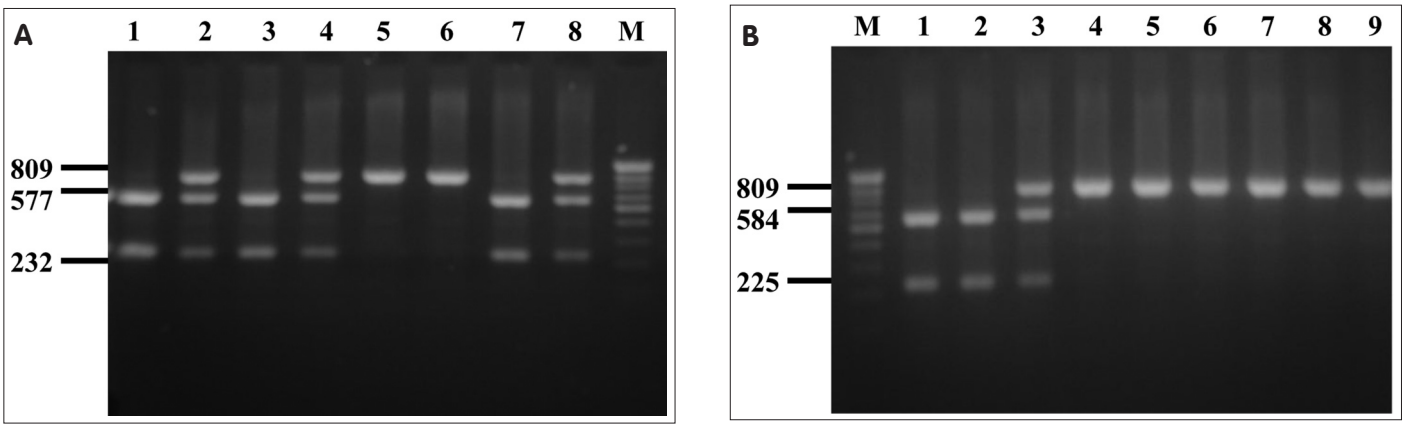

Figure 1. Gel electrophoresis showing PCR-RFLP analysis of VDBP exon 11 SNPs
A) Detection of Asp416Glu polymorphism after HaeIII digestion: fragments in lanes 1,3,7 (577 and 232 bp) indicates homozygote (Glu/Glu) genotype; lanes 5 and 6 showing a single band (809 bp) of undigested PCR product indicates homozygote (Asp/Asp) genotype; lanes 2,4,8: heterozygote (Asp/Glu) genotype (809, 577 and 232 bp).
(B) After StyI digestion for detection of Thr420Lys polymorphism, lanes 1,2 showing 2 bands (584 and 225 bp) indicate homozygote (Lys/Lys) genotype; the intact band (809 bp) in lanes 4-9 indicates Thr/Thr homozygote. Lane 3: individual with heterozygous pattern.

Table 1. VDBP genotypes distribution and allele frequencies in T1D patients versus healthy control subjects

	VDBP SNP	Control (n=65) No. (%)	T1D (n= 59) No. (%)	OR (95% CI)§	P value
Codon 416	Genotype				
	Glu/Glu	16 (24.6)	24 (40.7)	2.1 (0.98-4.52)	0.06
	Glu/Asp	40 (61.5)	28 (47.5)	0.57 (0.28-1.15)	0.12
	Asp/Asp	9 (13.9)	7 (11.8)	0.84 (0.29-2.41)	0.74
	Allele				
	Glu	72 (55.4)	76 (64.4)	1.46 (0.87-2.43)	
	Asp	58 (44.6)	42 (35.6)	0.69 (0.41-1.14)	0.15
Codon 420	Genotype				
	Thr/Thr	49 (75.4)	47 (79.7)	1.3 (0.55-2.99)	0.57
	Thr/Lys	16 (24.6)	10 (16.9)	0.63 (0.26-1.51)	0.29
	Lys/Lys	0	2 (3.4)		
	Allele				
	Thr	114 (87.7)	104 (88.1)	1.04 (0.49-2.24)	
	Lys	16 (12.3)	14 (11.9)	0.96 (0.45-2.06)	0.91

§OR (95% CI) = Odds Ratio (95% Confidence Interval). OR was calculated between cases and controls for specific genotype and allele frequency in relation to all the other genotypes except for Lys/Lys genotype which was not found in controls

Table 2. VDBP Haplotype frequencies in T1D patients and healthy control subjects

Haplotype	Control N (%)	T1D N (%)	OR (95% CI) §	P value
Gc-1f	42 (32.3)	28 (23.7)	0.65 (0.37-1.14)	0.13
Gc-1s	72 (55.4)	76 (64.4)	1.46 (0.87-2.43)	0.14
Gc-2	16 (12.3)	14 (11.9)	0.96 (0.45-2.06)	0.92

§OR (95% CI) = Odds Ratio (95% Confidence Interval). OR was calculated between cases and controls for specific genotype and allele frequency in relation to all the other genotypes

either genotype distribution or allelic frequencies in T1D compared to control subjects. No single genotype or allele was associated with an altered risk for T1D. Further analysis for the haplotypes constructed by these 2 polymorphisms was shown in Table 2. The three common haplotypes all had similar frequencies in non-diabetic control subjects and T1D patients.

Discussion

VDBP is the major transporter of 1,25(OH)2D3 and facilitates its peripheral bioavailability. Apart from transporting vitamin D, VDBP has been shown to influence the immune system through its conversion to a macrophage-activating factor by the activity of T- and B- lymphocyte enzymes (21). Furthermore, VDBP can serve as a co-chemotaxin for C5a and C5a des Arg. In this role, VDBP acts as a co-chemotactic factor in facilitating the chemotaxis of neutrophils and monocytes during the inflammation process (reviewed in 22). Recently, Thrailkill et al. (23) have shown exaggerated urinary excretion of VDBP in T1D patients which suggests a contribution of VDBP to vitamin D deficiency in this disease.

It has been suggested that SNPs in exon 11 of the VDBP gene generate functionally different proteins and that such differences affect circulating vitamin D concentrations (24). Moreover, the serum concentration of VDBP was reported to depend on its Gc phenotype (25).

Few studies have examined the relationship between VDBP polymorphism and risk of T1D. An earlier study among white Americans showed a borderline association of VDBP protein variants with T1D (15). Later, Pani et al. (16) failed to show such contribution to the disease in Germans. However, Ongagna and his colleagues, in their two reports (17, 26) confirmed such correlation in French Caucasians. They showed that the frequency of the distribution of VDBP alleles in codon 416 differed between diabetic subjects and controls and that the Glu/Glu genotype was significantly increased in T1D patients. However, their study failed to show an evidence of an association between the VDBP allele or genotype in codon 420 and T1D. We were not able to demonstrate any association between VDBP polymorphisms and risk of T1D in our patients. Although Glu/Glu genotype frequency was higher in T1D patients than in controls, however, this did not reach statistical significance (p= 0.06)

The apparent discrepancies between these studies could be a result of the effect of ethnic differences related to the distribution of VDBP polymorphisms in the different populations. This was demonstrated in other autoimmune diseases. For example, multiple sclerosis (MS) has been shown to be associated with Gc1f haplotype of VDBP protein in Icelandic patients while with Gc2 in Italian MS patients (27, 28). However, there was no correlation between MS and the distribution of VDBP variants in both Canadian and Japanese patients (29, 30). The VDBP gene Lys allele at codon 420 was reported to confer susceptibility to Graves' disease in the Polish population, while both exon 11 polymorphisms were not associated with Graves' disease in German patients (31, 32). Moreover, it was suggested that the VDBP 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.

Under physiological conditions, only 2% of VDBP is saturated with vitamin D metabolites (33). Thus, slight alterations in its affinity toward 1,25(OH)2D3 might not have any functional effect at all on the pathogenesis of T1D.

In conclusion, our data indicates that the two polymorphisms of the VDBP gene studied were not associated with T1D in a sample of Egyptian children, suggesting that it may not have a major effect on the susceptibility to T1D in this population. Up to our knowledge, this is the first study on the VDBP gene in an Egyptian population. Individually, biallelic SNPs have a low informational content. In a situation where the gene effect is very weak, and thus difficult to detect, a meta-analysis or a very large study group is required to prove the association. Further research on gene polymorphisms present in Egyptian patient groups is needed.

Conflict of Interest

None of the authors have any conflict of interest with regard to this manuscript.

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