Vitamin D Level and Fok-I Vitamin D Receptor Gene Polymorphism in Egyptian Patients with Type-1 Diabetes

[1]Elham O Hamed, [2]Amal M Abdel-Aal, [3]Amal K Norel Din, [3]Mervat MA Atia

[1]Clinical Pathology Department and [3]Internal Medicine Department, Faculty of Medicine, Sohag University, [2]Clinical Pathology Department, Faculty of Medicine, Assiut University, Egypt.

Many cellular, preclinical and observational studies support a role of vitamin D in pathogenesis of type-1 diabetes mellitus (DM). The vitamin D receptor (VDR) locus has been studied in different populations for association with susceptibility to immune-mediated diseases, but with inconsistent findings in type-1 DM. This study aimed to investigate vitamin D status in patients with type-1 DM. We examined the frequency of VDR FokI (rs10735810) gene polymorphism, and its association with serum 25-hydroxyvitamin D (25(OH) D) level in Egyptian patients with type-1 DM. 132 children with type-1 DM and 40 age and sex matched healthy control subjects were studied. VDR FokI polymorphism was assessed using polymerase chain reaction and restriction fragment length polymorphism (RFLP) analysis. Diabetic children demonstrated lower circulating levels of 25(OH) D than the controls (13.4±7.6 vs 32.1±3.8ng/ml) (*P*<0.01). Patients with deficient 25(OH) D showed lower calcium levels and higher HbA$_{1c}$% than those with sufficient levels (8.1±2.1 versus 9.1±1.6 mg/dl & 9.9±2.5 versus 8.1±1.4%, respectively (*P*<0.05). There was no significant difference in the genotype distribution or the allele frequencies of VDR FokI between patients and controls. The odds ratio (OR) was 1.08 (*P*=0.76), and the 95% confidence interval (CI) ranged from 0.64-1.85. The diabetic carriers of the ff genotype showed low serum levels of 25(OH) D and calcium when compared with the carriers of the F allele (9.1±4.4 vs 13.1±7 and 13.9±6.09 ng/ml & 8.1±2.1 vs 9.1±1.1 and 9.3±1.2 mg/dl, respectively) (*P*<0.05). In conclusion, 84.8% of children with type-1 DM have low circulating levels of 25(OH) D. These patients have poor glycemic control (56.06%) than those with sufficient levels of 25(OH) D. FokI polymorphism of VDR gene is associated with vitamin D deficiency but has no significant role in susceptibility to type-1 diabetes.

Type-1 diabetes mellitus (DM) is a multifactorial disease caused by interactions between many genetic and environmental factors. It is caused by autoimmune destruction of pancreatic β-cells (Chen *et al.,* 2011) and characterized by insulin secretion deficiency and ketosis-prone hyperglycemia (Barrett *et al.,* 2009). The incidence of type-1 diabetes varies with geographic patterns (Tatiana *et al.,* 2010). Multiple components in the metabolic pathway of vitamin D may be altered in type-1 DM (Assy *et al.,* 2012). The parallel rise in incidence of both type-1 DM and vitamin D deficiency raises the possibility that vitamin D may play a role in the pathogenesis of type-1 DM (Mathieu *et al.,* 2005). Evidence from basic, clinical, and epidemiological studies provides a rationale for this hypothesis (Zeitz *et al.,* 2003; Gysemans *et al.,* 2005; Adams *et al.,* 2007). Vitamin D is a potent modulator of the immune system and is involved in the regulation of cell proliferation and differentiation. Vitamin D is an effective immuno-suppressant via inhibition of lymphocyte activation and cytokine production and prevents or markedly suppresses the development of several autoimmune diseases in animal models (Nagpal *et al.,* 2005). Moreover, low serum levels of 25(OH) D3 and 1, 25 (OH)2D3 correlate with an impaired function of the immune system and have been associated with this disease (Pozzilli *et al.,* 2005). It was

reported that; administration of vitamin D protects against the development of insulitis and type-1 diabetes in non-obese diabetic mice (Mathieu *et al.,* 1994). In humans, epidemiological studies indicated that dietary vitamin D supplementation during early childhood decreases the risk of type-1 DM (Hypponen *et al.,* 2001) and that maternal intake of vitamin D during pregnancy may have a protective effect on the appearance of islet autoantibodies in offspring (Fronczak *et al.,* 2003).

Because vitamin D exerts its effects through the vitamin D receptor (VDR), the VDR gene has become a candidate susceptibility gene for type 1 diabetes. VDR gene is located on chromosome 12 and contains 9 exons (Li *et al.,* 2009). According to Davis (2008), over 470 VDR single nucleotide polymorphisms (SNPs) of this gene were investigated. However, four common polymorphisms are known including: rs10735810 or FokI in exon2, rs1544410 or BsmI in intron 8, rs731236 or TaqI in exon9, and rs7975232 or Apa1 in intron 8. Variants of the VDR gene have been associated with susceptibility to several autoimmune processes. The roles of the VDR gene polymorphisms depend on their locations (Slattery, 2007). FokI polymorphism is within the DNA binding domain, near the 5′ end, and the rest of the SNPs are in the 3′-UTR region within the ligand binding domain. The FokI polymorphism creates an alternative ATG initiation codon in exon 2 leads to a 3 amino-acids longer VDR protein by directly introducing a start codon. A functional impact of this polymorphism on the immune response has been demonstrated (Colin *et al.,* 2000; van Etten *et al.,* 2007). However, VDR gene SNPs influence on VDR expression differ in different populations.

In this study we investigated vitamin D status in patients with type-1 DM, investigated the frequency of VDR FokI gen

polymorphisms, its susceptibility to type-1 DM and its association with serum levels of 25(OH) D in Egyptian population. In order to further evaluate the consequences of the polymorphism for vitamin D metabolism.

Subjects and Methods

Subjects and study design

In a case control study, we evaluated 132 children patients (64 males and 68 females) who had type-1 DM, according to American Diabetes Association criteria (American Diabetes Association 2010). They were attending the Diabetes clinic at Sohag University Hospital from June 2011 to December 2012. Their age ranged from 2-15 years (mean±SD 8.5±3.3 years). The mean duration of diabetes was 3.1±2.7 years. Also, 40 non diabetic children attending outpatient clinic with unrelated complains, apparently healthy and they had no family history of diabetes mellitus were included as a control group (18 males and 22 females). Their age ranged from 4-15 (9±1.5 years). All participants were subjected to full history taking to identify family history of DM and age of onset of the disease, and clinical examination. Exclusion criterion: subjects taking vitamin D or calcium therapy. The study conducted through cooperation between the clinical pathology department, Sohag University and Clinical Pathology department, Assiut University. It was approved by the faculty committee for research ethics. Informed consent was obtained from the subjects' parents.

Methods

Blood samples were obtained from patients and controls after 8-12 hours of fasting, for laboratory analysis and VDR genotyping. Whole blood on EDTA coated tubes used for the extraction of genomic DNA and glycosylated hemoglobin (HbA_{1c}). Serum glucose, serum calcium, serum phosphorus, liver and kidney functions tests were determined by autoanalyzer Cobas c 311 (Roche/Hitachi cobas c systems). HbA_{1c} was determined by ion-exchange high-performance liquid chromatography (HPLC) (The BioRad D10 analyser). The D-10 Hemoglobin A_{1c} Program is based on chromatographic separation of HbA_{1c} on a cation exchange cartridge. The samples are automatically diluted on the D-10 and injected into the analytical cartridge. The D-10 delivers a programmed buffer gradient of increasing ionic strength to the cartridge, where the hemoglobins are separated based on their ionic interactions with the cartridge material. The separated hemoglobins then pass through the flow cell

of the filter photometer, where changes in the absorbance at 415 nm are measured (D-10 Hemoglobin A_{1c} program 2004).

Serum 25-hydroxyvitamin D test was performed using fully automated ARCHITECT instrument (Abbott Diagnostics Division, Chicago) based on Chemiluminescent microparticle immunoassay (CMIA). Sample and pre-treatment reagents are combined. An aliquot of the pre‑ treated sample is combined with assay diluent and paramagnetic anti-vitamin D coated microparticles to create a reaction mixture. Vitamin D present in the sample binds to anti-vitamin D coated microparticles. After incubation a biotinylated vitamin D anti-Biotin acridinium labeled conjugate complex is added to the reaction mixture and binds to unoccupied binding sites of the anti-vitamin D coated microparticles. After washing, pre-trigger and trigger solutions are added to the reaction mixture. The resulting chemiluminescent reaction is measured as relative light units (Carter *et al.,* 2004).

Determination of FokI genotypes by PCR-RFLP

FokI polymorphism was assessed by polymerase chain reaction (PCR) technology and restriction fragment length polymorphism (RFLP) analysis. Genomic DNA was extracted from 200 µl whole blood by protease digestion using QIAamp blood mini kit (QIAGEN, Germany). DNA was amplified with specific primers: (Forward) 5′- AGC TGG CCC TGG CAC TGA CTC TGC TCT-3′ and (Reverse) 5′-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3′ (Panierakis *et al.*, 2009). The 25 µl reaction mixture contained PuReTaq Ready to go PCR beads supplied by GE health Care, 50 pmol of each primer and 50 ng of genomic DNA. Amplification was done according to the following protocol: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 15 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec; and final extension at 72°C for 7 min. The amplified products (265 bp) were incubated for one hour at 37°C with FokI restriction enzyme (Jena Bioscience, Germany) in a 50 µl mixture containing 2 units of the enzyme, 5 µl of 10X buffer and 10 µl of the product. After incubation, the reaction was stopped by heat inactivation for 20 min. at 65°C. The resulting fragments were visualized by electrophoresis on 2.5% agarose gel under UV light. In the presence of the FokI restriction site, digestion with the FokI restriction enzyme generated two fragments, of 196 and 69 bp, respectively. The presence of a restriction site was represented by lowercase (f) and its absence by the uppercase (F) allele.

Statistical Analysis

Statistical analyses were performed using SPSS version 19. Data were summarized as mean ± SD, range or percentage. Allele and genotypic frequency was calculated by direct gene counting method. Comparison of the different genotypes was done by using Chi square test. Odd's ratios were calculated with a 95% confidence interval limit. Clinical characteristics of patients with FokI genotypes were compared using independent t test. One way analysis of variance (ANOVA) test was used to determine the significance of variables when comparing more than two groups. *P*-value <0.05 was considered significant.

Results

Clinical characteristics and laboratory variables of the studied groups were demonstrated in Table 1. Blood glucose and HbA_{1c} levels were significantly higher in type-1 diabetes patients than control group (*P*<0.01 for each). Patients showed poor glycemic control (HbA_{1c} ≥8.0%) were 56.06%. Positive family history of diabetes was found in 27.3% of the patients. Serum levels of calcium and 25(OH) D were significantly lower (*P*<0.01 for each), while inorganic phosphorus was significantly higher (*P*<0.01) in type-1 DM patients than controls. In the control group, the level of 25(OH) D ranged from 25-42 ng/ml. Patients were classified into two groups according to the lower limit of control group; deficient group (<25 ng/ml), and sufficient group (≥25 ng/ml). 84.8% of patients had 25(OH) D levels <25 ng/ml and 15.2% of them had 25(OH) D levels ≥25 ng/ml. We compared different laboratory parameters between the two groups. We found that; Patients with deficient 25(OH) D showed lower serum calcium levels (*P*<0.05) and higher HbA_{1c}% (*P*<0.05) than patients with sufficient 25(OH) D (Table 2).

Table 1. Clinical and Laboratory Characteristics of Children with Type-1 Diabetes and Controls

	Type 1 DM patients (n=132)	Healthy Controls (n=40)	P-value
Age (years)	8.5±3.3	9±1.5	NS
Males %	48.5%	45%	NS
Age at diagnosis (years)	5.9±2.3	-	-
Duration of diabetes (years)	3.1±2.7	-	-
Family history of diabetes	27.3%	-	-
S. glucose (mg/dl)	215.1±130.5	87±8.9	< 0.01
HbA$_{1c}$%	8.9±2.4	5.1± 0.9	< 0.01
25(OH) D(ng/ml)	13.4±7.6	32.1 ± 3.8	< 0.01
Calcium (mg/dl)	7.7±2.1	9.8±0.9	< 0.01
Phosphorus (mg/dl)	4.9±1.6	3.6±0.9	< 0.01

-Data were presented as mean±SD or as %.

-P<0.05 was considered significant.   -NS= not significant.

Table 2: Laboratory Characteristics of Patients with Type 1 Diabetes according to 25(OH) D Levels

	Vit D deficient group 25(OH)D <25ng/ml N=112	Vit D sufficient group 25(OH)D ≥25ng/ml N=20	P-value
S. glucose(mg/dl)	225.1±99.5	215.1±120.1	NS
HbA$_{1c}$%	9.9±2.5	8.1±1.4	< 0.05
Calcium(mg/dl)	8.1±2.1	9.1±1.6	< 0.05
Phosphorus(mg/dl)	5.1±1.06	4.7±1.2	NS

-Data were presented as mean±SD or as %.
-P<0.05 was considered significant.   -NS= not significant.

FokI restriction patterns of the various genotypes are represented in Figure 1. FF indicates absence of FokI restriction site (one band at 265 bp) and ff indicates presence of FokI restriction site (two bands at 196 and 69 bp). The presence of three bands after treatment with restriction enzyme at 265, 196, and 69 bp indicates the heterozygous Ff genotype.

The genotype and allele-frequencies of the FokI VDR polymorphism in type-1 DM patients and controls are described in Table 3. There was no statistically significant difference in the distribution of genotypes or alleles between patients and controls. The odds ratio was 1.08 (P=0.76), and the 95% confidence interval ranged from 0.64 - 1.85. Laboratory parameters in patients carrying different genotypes were compared. Carriers of the ff genotype showed low serum levels of 25(OH) D and calcium compared with the carriers of the F allele ($p < 0.05$ for each) (Table 4).
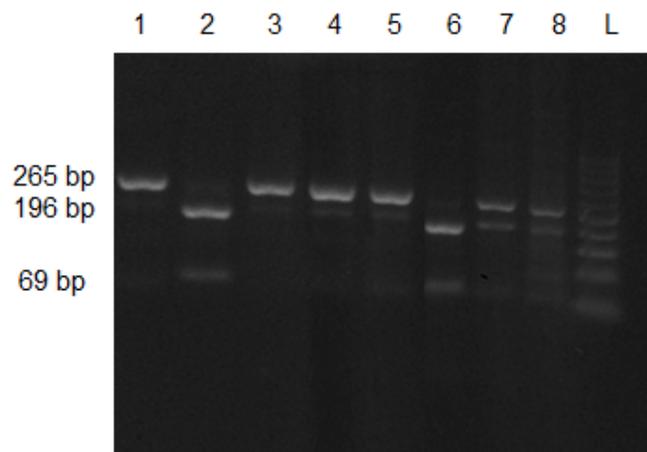
Figure 1. Determination of *FokI* Polymorphism by PCR-RFLP. Restriction Endonuclease Digestion for FokI Polymorphism. FF Indicates Absence of FokI RE site, ff-presence of FokI RE site. Lanes 1, 3, 4 and 5 -FF (265 bp), Lane 2 and 6- ff (196 bp and 69 bp), Lane 7 and 8- Ff (265 bp, 196 bp and 69 bp), L-DNA ladder.

Table 3. Distribution of Genotype and Allele Frequency in Patients and Control Group

Genotype	Patients n=132	Controls n=40	P-value	OR (95% CI)
FokI Polymorphism				
Allele Frequency				
F	140 (53%)	44 (55%)	NS	0.92 (0.54-1.57)
f	124 (47%)	36 (45%)	NS	1.08 (0.64-1.85)
Genotype Frequency				
FF	24 (18.2%)	8 (20%)	NS	0.89 (0.34-2.51)
Ff	92 (69.7%)	28 (70%)	NS	0.99 (0.41-2.24)
ff	16 (12.1%)	4 (10%)	NS	1.24 (0.36-5.42)

P>0.05 is not significant (NS)

Table 4. Laboratory Parameters in Type 1 Diabetes Patients Classified according to *FokI* Polymorphism.

	Genotype			
	FF	Ff	ff	p-value
	(n= 24)	(n= 92)	(n= 16)	
S. glucose (mg/dl)	199±110.2	205.1±99.1	202.1±105.1	NS
HbA$_{1c}$%	8.9±1.9	9.1±1.5	9.3±2.2	NS
25(OH) D (ng/ml)	13.9±6.09	13.1±7.01	9.1±4.4	< 0.05
Calcium (mg/dl)	9.3±1.2	9.1±1.1	8.1±2.1	< 0.05
Phosphorus (mg/dl)	5.1±0.9	4.8±1.2	4.9±1.1	NS

-Data were presented as mean±SD or as %.

-*P*<0.05 was considered significant.   -NS= not significant.

Discussion

The prevalence of type-1 DM in childhood is increasing with a worldwide annual increase estimated at 3% (Ambler *et al.,* 2006). Environmental factors play an important role in the development and progression of systemic autoimmune diseases along with susceptible genetic and hormonal background. Type-1 DM is an autoimmune disease and environmental factors contribute to its development (Rewers *et al.,* 2004). Several studies have focused on the association between candidate genes and development of type-1 diabetes. Previous studies of the relation between vitamin D receptor gene polymorphism and risk of type-1 diabetes have produced inconsistent results in different populations. We studied the distribution of a common polymorphism (FokI) in patients with type-1 diabetes and its relation with vitamin D status in these patients.

Vitamin D stores are derived from either dietary intake or cutaneous synthesis following ultraviolet irradiation. Vitamin D from either source undergoes 25-hydroxylation in the liver to form 25-hydroxy vitamin D. Optimal vitamin D status is important to human health, and there is a consensus that serum 25(OH) D should be used to assess vitamin D status because it reflects combined dietary supply and dermal production (Cashman 2007).

The present study demonstrated that 25(OH) vitamin D and calcium levels were significantly decreased in diabetic patients compared to controls. While inorganic phosphorus was significantly higher in type-1 DM patients than in controls. Lower serum calcium levels may be the result of the decrease in duodenal calcium absorption and an increase in its urinary excretion in diabetic patients. The high inorganic phosphorus serum level observed in the studied patients could be explained by physiological feedback mechanism to reduced serum calcium in order to maintain the solubility product constant (Carnevale *et al.,* 2004).

Among the 132 participants with DM type-1, 84.8% had deficiency of vitamin D. The above-mentioned findings are consistent with a number of studies in Egyptians and other populations (Pozzilli *et al.,* 2005; Littorin *et al.,* 2006; Svoren *et al.,* 2009; Hamed *et al.,* 2011; Hussein *et al.,* 2012). The lower levels of 25(OH) D found in diabetic patients compared to control subjects support the idea

that vitamin D deficiency may be an important factor behind the development of type-1 diabetes, perhaps with an immunological background (Zemunik *et al*., 2005). Svoren *et al*. (2009) reported significant vitamin D deficiency in 76% of children with type-1 diabetes. The reduced 25(OH) D serum level in type-1 DM can be attributed to decreased synthesis of vitamin D-binding protein by the liver (Carnevale *et al.,* 2004).Vitamin D deficiency was implicated in the impairment of insulin synthesis and secretion (Zeitz *et al.,* 2003), while vitamin D supplementation has been demonstrated to attenuate cytokine-mediated pancreatic beta-cell destruction (Gysemans *et al.,* 2005). Moreover, the reported inverse correlation between sunlight exposure and type-1 DM incidence is consistent with the hypothesis that vitamin D status modulates disease susceptibility (Pozzilli *et al.,* 2005).

In this study, there was significant association between the concentrations of 25(OH) D & HbA_{1c} among the studied type-1 DM patients; patients with deficient vitamin D (<25 ng/ml) had higher HbA_{1c}% than those with sufficient vitamin D (≥25 ng/ml) indicating poor glycemic control in these patients. Vitamin D is the major regulator of calcium absorption, it mediates the activity of β-cell calcium-dependent endopeptidases and thus, promotes the conversion of proinsulin to insulin and increases insulin output. In peripheral insulin-target tissues, vitamin D enhances insulin action via regulation of the calcium pool (Tuorkey & Abdul-Aziz 2010).

Receptors for 1, 25 (OH) D are expressed in antigen-presenting cells and T-cells as well as in pancreatic beta cells (Adams *et al*., 2007). The vitamin D receptor gene locus has been studied in different populations for association with susceptibility to immune-mediated diseases but with inconsistent findings in type-1 diabetes mellitus (Guo *et al.,* 2006). In the studied patients, we didn't find evidence of allelic or genotypic association of the FokI SNPs of the VDR gene with type-1 diabetes mellitus. Lemos *et al.* (2008) and Zhang *et al.* (2012) also reported no statistical evidence of an association between the FokI polymorphism and overall type-1 DM risks. However, another study in southern European population found that FokI polymorphism of the VDR gene is associated with type-1 DM prevalence (Panierakis *et al*., 2009).The frequency of VDR FokI genotype ff was 12.1% in the studied patients and 10% in control group. Japan, has the frequency of FokI (ff genotype 11%) (Yokota *et al.,* 2002); Hungary 17%; and Spain, 12% (Gyorffy *et al.,* 2002; AudÎ *et al.,* 2004) which were similar to that observed in the present study. On the other hand, the frequency of this genotype was markedly different (ff 7-50%) in Finland, where the highest incidence of type-1 DM of the world is observed (Turpeinen *et al.,* 2003).

In the present study, the diabetic carriers of the ff genotype showed low serum levels of 25(OH) D compared with the carriers of the F allele (*P*<0.05). This is consistent with another study done by López *et al.* (2008) which found low levels of 25(OH) D in carriers of the ff genotype. Vitamin D supplementation may have a role in pharmacological management of diabetes mellitus or as a preventive therapy in some subsets of type-1 DM patients (Assy *et al.,* 2012).

The VDR FokI polymorphism has a functional role on the immune system. Van Etten *et al.* (2007) observed in vitro that lymphocytes without VDR FokI polymorphism proliferated more strongly, with a more active immune response. Also, monocytes and dendritic cells without this polymorphism produced higher levels of IL-12p70 protein after stimulation. This is the major cytokine that induces a T–helper 1

response, resulting in cell destruction (Kukreja & Maclaren 1999). In this study we did not aim to evaluate the immune response, and further studies are necessary to determine the relationship between cytokines and VDR genotypes in Egyptian type-1 DM patients.

In conclusion, children with type-1 DM (84.8%) have low circulating levels of 25(OH) D. These patients have poor glycemic control (56.06%) than those with sufficient levels of 25(OH) D as determined by higher HbA_{1c}% in these patients. FokI polymorphism of VDR gene is associated with vitamin D deficiency but has no significant role in susceptibility to type-1 diabetes. Monitoring of vitamin D status is recommended in routine assessment of type-1 DM. Further genomic investigations to other common single nucleotide polymorphisms in the VDR gene and susceptibility to type-1 diabetes in Egyptian population are recommended.

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