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Lack of association between *FokI* polymorphism in vitamin D receptor gene (*VDR*) & type 2 diabetes mellitus in the Tunisian population

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***Background & objectives*: The impact of several environmental and genetic factors on diabetes is well documented. Though the association between the vitamin D receptor (*VDR*) gene polymorphisms and type 2 diabetes mellitus (T2DM) has been analyzed in different ethnic groups, the results have been inconsistent. The aim of this study was to evaluate the possible association between *VDR FokI* polymorphism and genetic susceptibility to T2DM in Tunisian population.**

***Methods*: A total of 439 unrelated patients with T2DM and 302 healthy controls were included in the study. Genomic DNA was extracted from blood and genotyped for the single nucleotide polymorphism (SNP) of *FokI* (T/C: (rs2228570) by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) analysis.**

***Results*: The genotype distribution and the relative allelic frequencies for the *FokI* polymorphism were not significantly different between T2DM and controls: in T2DM patients the frequencies of the CC, CT, and TT genotypes were 52.6, 41.0, and 6.1 per cent, respectively, and in controls the genotype frequencies were 55.6, 38.7, and 5.6 per cent, respectively. In our study, the TT genotype of the *FokI* polymorphism was not associated with T2DM (OR =1.19, 95% CI 0.63 - 2.25, *P*=0.577).**

***Interpretation & conclusions*: Our study showed no significant association of the *FokI* polymorphism in the vitamin D receptor gene with type 2 diabetes mellitus in Tunisian population.**

Key words *FokI* - single nucleotide polymorphism - T allele - type 2 diabetes - VDR

The incidence of type 2 diabetes mellitus (T2DM) is increasing at an alarming rate worldwide. T2DM is a multifactorial metabolic disorder, influenced by both genetic and environmental factors and exhibits a wide range of disparities among different ethnic groups[1-5]. The identification of genes predisposing to T2DM could provide means to better understand the pathogenesis of the disease and result in better prevention and treatment.

Vitamin D deficiency is shown to be associated with glucose intolerance, insulin resistance, metabolic syndrome, and increase risk for diabetes[6]. Vitamin D

exerts its actions on target tissues through its binding to the cytosolic/nuclear vitamin D receptor (VDR), which is a member of the steroid/thyroid hormone receptor family that functions as a transcriptional activator of many genes. The gene encoding the VDR is located on chromosome 12q13.1[7], contains 11 exons and spans approximately 75 kb. Several polymorphisms have been identified in the *VDR* gene, and their functional significance and potential effects on disease susceptibility have been investigated[8]. Among these polymorphisms, the *FokI* polymorphism (ATG→ACG) located in the exon2 of the gene is the only known locus affecting the structure of the VDR protein produced. The T allele encodes a 427 amino acid protein while the C allele encodes a 424 amino acid protein[9-11]. The shorter VDR protein variant seems to function more effectively and further increases its capacity of binding 1,25-dihydroxyvitamin D[12], and the relatively higher level of vitamin D, in turn, can reduce the risk of T2DM by enhancing pancreatic b-cell secretion function and improve insulin resistance[13]. This biological mechanism could explain the association between the T allele of *FokI* polymorphism and susceptibility to T2D.

Several epidemiologic studies have examined the association of the *FokI* polymorphism of the *VDR* gene and T2DM, and the results are inconsistent across different populations[14-19]. The aim of the present study was to analyze the association between the *FokI* polymorphism of the *VDR* gene and TD2M in a sample of the Tunisian population.

Material & Methods

A total of 741 individuals were included in this case-control study. Four hundred thirty nine unrelated patients with T2DM (263 women and 176 men) with mean age of 55.9 ± 9.7 yr were enrolled at the Department of Endocrinology of Rabta University Hospital of Tunis, Tunisia, from January 2007 to July 2009. Diabetes was diagnosed according to the World Health Organization (WHO) criteria[20]. Diabetes was defined as hyperglycaemia, requiring antidiabetic drugs or testing blood glucose level ≥ 7.0 mmol/l or a 2-h post-challenge glucose level ≥ 11.1 mmol/l. The control group consisted of 302 unrelated individuals (190 women and 112 men) with a mean age of 49.3 ± 9.6 yr, who underwent a medical checkup in our hospital. The exclusion criteria included fasting blood glucose levels of more than 100 mg/dl or a family history of diabetes. Diabetic patients with complications of malignancies were also excluded. Patients and controls were homogeneous Tunisian Arab lineage from Northern Tunisia. Informed written consent was obtained from all participants and the design of the study was approved by the local ethics committee.

Infromation on demography, socio-economic status, education, lifestyle and mental health was collected. Weight and height were measured. Body mass index (BMI; kg/m^2) was calculated and obesity was defined as BMI ≥ 30 kg/m^2 for both genders[21]. Hypertension was defined as systolic blood pressure (SBP) ≥ 140 mm Hg and/or diastolic blood pressure (DBP) ≥ 90 mm Hg, or the use of antihypertensive drug treatment or a combination of these. Dyslipidaemia was defined as a total cholesterol (TC) level > 6.47 mmol/l and /or triglyceride level (TG) > 2.26 mmol/l. Smoker definition included both ex-smokers and active smokers. A daily consumption of more than five cigarettes was considered a smoking habit[22].

Laboratory analysis : Blood samples (10 ml) were obtained after an overnight fast. Blood glucose, TC, TG and high-density lipoprotein cholesterol (HDL-C) were measured in the hospital laboratory on a Hitachi 912 analyzer (Roche Diagnostics, Mannheim, Germany) using the respective reagent kits. Low-density lipoprotein cholesterol (LDL-C) was calculated according to the equation of Friedwald *et al*[23].

DNA analysis: Genomic DNA was prepared from white blood cells by phenol extraction[24]. Genotyping of the *FokI* (T/C) (rs2228570) *VDR* polymorphism was performed with the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method as described previously[25]. Briefly, a fragment of 265 bp including the *Fok1* polymorphism was amplified using two oligonucleotides[25]:

Forward:
5'-AGCTGGCCCTGGCACTGACTCTGCTCT-3'

Reverse:
5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3'

The PCR product was digested by the restriction enzyme *FokI* (MBI Fermentas, Vilnius, Lithuania) followed by electrophoresis on a 3 per cent agarose gel stained with ethidium bromide and visualized using ultraviolet illumination. The wild type homozygote (CC), heterozygote (CT) and mutant homozygote (TT) showed one band (265 bp), three bands (265, 196 and 69 bp) and two bands (196 and 69 bp), respectively

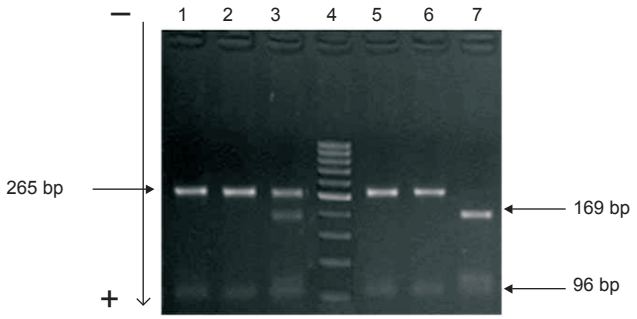

Figure. The *FokI* digestion profiles of the 265 bp PCR product of the *VDR* gene homozygous (CC) individuals showing an undigested 265 bp product (lands 1, 2, 5 and 6); heterozygous (CT) individuals showing 265, 196, and 69 bp fragments (lane 3); homozygous (TT) showing 196 and 69 bp fragments (lane 7). GeneRuler DNA ladder (1000-50 bp) (lane 4).

(Figure). The genotype of each sample was determined by two technicians working independently and for quality control, 20 per cent of the samples were selected at random for repeated genotyping and concordance was 100 per cent.

Statistical analysis: Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 15.0 for windows, SPSS Inc., Chicago, IL, USA). Distributions of continuous variables in groups were expressed as mean ± SD, and compared with unpaired Student's *t* test. c^2 test was used to test for departures from Hardy-Weinberg equilibrium and to compare genotype distributions between groups. Odds ratio (OR) at 95% confidence interval (CI) was determined to describe the strength of association by logistic regression model.

Table I. Demographic and clinical characteristics of the study population

	T2DM (n = 439)	Controls (n = 302)
Age (yr)	55.94 ± 9.76***	49.38 ± 9.63
BMI (kg/m^2)	30.07 ± 5.36***	27.88 ± 5.59
Dyslipidaemia (%)	34.9***	20.0
Hypertension (%)	562***	16.3
Obesity (%)	43.8***	28.4
Smokers (%)	12.6**	21.6
Fasting glucose (mmol/l)	9.76 ± 4 .38***	4.99 ± 0.49
Total cholesterol (mmol/l)	4.99 ± 1.03	4.92 ± 0.95
LDL cholesterol (mmol/l)	3.19 ± 0.91**	2.97 ± 0,82
HDL cholesterol (mmol/l)	1.13 ± 0.44***	1.29 ± 0.33
Triglycerides (mmol/l)	1.55 ± 0.93*	1.40 ± 0.92

T2DM, type 2 diabetes mellitus; LDL, low density lipoprotein; HDL, high density lipoprotein
P *<0.05, **<0.01, ***<0.001 compared to controls

Results

The clinical characteristics of the study population are shown in Table I. There was significant differences for age ($P<0.001$), and BMI ($P<0.001$), and the frequencies of dyslipidaemia ($P<0.001$), hypertension ($P<0.001$) and cigarette smoking ($P<0.01$) between T2DM and control groups. The baseline TG ($P<0.05$) and LDL-C concentrations were higher in the T2DM patients ($P<0.01$). In addition, T2DM patients presented lower HDL-C levels ($P<0.001$). The genotype distribution and the relative allele frequency of the *FokI* polymorphism at the *VDR* gene in T2DM patients and controls are shown in Table II. Genotype frequencies did not deviate from the Hardy-Weinberg equilibrium in control individuals and T2D patients. The frequencies of the CC, CT and TT genotypes among control group were 55.6, 38.7, and 5.6 per cent, respectively whereas the corresponding frequencies among the patients were 52.6, 41.0 and 6.4 per cent, respectively.

No significant difference in polymorphism, genotype distribution and allele frequency was observed between patients and controls (Table II). When the samples were subgrouped by gender, no significant association was noted between T2DM patients and the controls (data not shown).

Discussion

Type 2 diabetes mellitus is a complex disease caused by complex interplay between environmental and genetic factors. Vitamin D is essential for the function of the endocrine pancreas, and the *VDR* gene may be involved in the pathogenesis and progression of T2DM. Several studies have examined the association of various *VDR* genetic variants and T2DM, and the results are inconsistent[26,27]. Our data showed that the *FokI VDR* polymorphism was not associated with T2DM in Tunisian population. The T allele frequency of *FokI VDR* polymorphism was similar between T2DM and control subjects. The *FokI* polymorphism, either singly or in combination with other *VDR* polymorphisms, has been investigated in a few studies on diabetes risk assessment and results

Table II. Genotype distribution and allele frequencies of *VDR-FokI* polymorphism

	T2DM (n = 439)	Controls (n=302)	Unadjusted odd ratios	*P* value
Genotypes	N (%)			
CC	231 (52.6)	168 (55.6)	1[a]	
CT	180 (41.0)	117 (38.7)	1.11 (0.82 - 1.52)	0.472
TT	28 (6.4)	17 (5.6)	1.19 (0.63 - 2.25)	0.577
Alleles				
C	0.73	0.75	-	
T	0.27	0.25	1.10 (0.86 - 1.41)	0.418

[a]Reference category; T2DM, type 2 diabetes mellitus

Table III. Associations with type 2 diabetes mellitus of the *VDR* gene in various sample populations

Authors	Population	Year	T2DM N	Controls N	OR (95% CI)	Significant results
Malecki *et al*[15]	Polish	2003	308	239	1.08 (0.85-1.37)	No
Bid *et al*[16]	Indian	2009	100	160	0.72 (0.49-1.06)	No
Al-Daghri *et al*[17]	Saudi	2012	368	259	0.80 (0.41-1.59)	No
Vedralova *et al*[18]	Czech	2012	116	118	1.12 (0.77-1.62)	No
Li *et al*[19]	Asian	2013	104	77	1.93 (1.23-3.04)	Yes
Mackawy *et al*[28]	Egyptian	2014	63	60	0.51 (0.37-0.72)	No
Errouagui *et al*[30]	Moroccan	2014	176	177	0.35 (0.14- 0.83)	Yes

N, number; T2DM, type 2 diabetes mellitus

were controversial (Table III). Bid *et al*[16] using *FokI*, *BsmI*, and *TaqI* demonstrated that there was no link between polymorphisms in the *VDR* gene and T2DM. Malecki *et al*[15] reported no significant difference in the distribution of the allele and genotype frequencies of the *FokI* polymorphism between 308 T2DM patients and 239 healthy controls from Poland similar to our study. However, in an Egyptian study, involving 63 patients with T2DM and 60 controls, the *FokI* variant was significantly associated with risk of T2DM only in patients with metabolic syndrome[28]. A meta-analysis of 10 studies involving 1562 cases and 1461 controls, showed that the FokI polymorphism was associated with an increased risk of T2DM (T vs. C: OR = 1.30, 95% CI = 1.17 - 1.45, $P<0.001$), especially in East Asians[14]. This was confirmed in another meta-analysis including five studies (1101 cases and 969 controls) of Li *et al*[19], which showed that allele T and variant homozygote TT of *FokI* variant were significantly associated with T2DM, especially in an Asian population (T vs. C: OR = 1.25, 95% CI = 1.10 - 1.41; TT vs. CC: OR = 1.48, 95% CI = 1.13 - 1.94). A meta-analysis by Yu *et al*[30] including 12 studies showed significant associations of *FokI* polymorphism with T2DM for three genetic models (TT vs. CC: OR = 1.57, 95% CI = 1.28 - 1.93, $P<0.001$; CT vs. CC: OR = 1. 54, 95% CI = 1.31 - 1.81, $P<0.001$; TT + CT vs. CC: OR = 1.57, 95% CI = 1.35 - 1.83 , $P<0.001$), especially in Chinese population. On the other hand, a protective effect of the *FokI* T allele against T2DM was reported by Errouagui *et al*[29] in a case-control study involving 176 patients with T2DM and 177 healthy controls subjects (OR = 0.35, 95% CI = 0.14 - 0. 83, $P=0.018$) in Moroccan population.

The discrepancies between the studies may be explained by the different allelic frequencies observed in different ethnic groups. For example, the C allele frequency was lower in Africans when compared to Caucasians and Asians[9]. Other explanation for the diversity of the results are selection criteria adopted for

patients and controls, in particular age, ethnicity, extent of disease, concomitant environmental risk factors like differences in the lifestyles (smoking, diet and physical activity) and the gene-gene and gene-environment interactions.

The present study had some limitations. First, the small size of patients and controls groups which may limit the power (60%) to detect the *FokI* polymorphism effect on T2DM. Second, our results were limited by the absence of both dietary information and plasma vitamin D concentrations for our study participants. Studies have shown that the association between *VDR* polymorphisms and disease can vary by either past sun exposure or vitamin D level[31]. Our study was based on estimates without adjusting for sun exposure or vitamin D intake. Third, polymorphisms of other *VDR* genotypes, i.e., *TaqI*, *ApaI*, and *BsmI*, and their possible interactions with *FokI* variants were not evaluated.

In conclusion, our study indicated that the *FokI* variant of the *VDR* gene was not associated with T2DM in the Tunisian population. It is possible that the effect of *FokI* variant on T2DM risk is specific to some particular ethnic populations. The present results require confirmation in further and larger studies in the Tunisian population.

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***Conflicts of Interest*:** None.

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