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

Imen Mahjoubi¹, Amani Kallel¹, Mohamed Hédi Sbaï¹, Bochra Ftouhi², Meriam ben Halima¹, Zeineb Jemaa¹, Moncef Feki¹, Hedia Slimane², Riadh Jemaa¹ & Naziha Kaabachi¹

¹Research Laboratory LR99ES11, Biochemistry Department, University of Tunis El Manar, Tunis & ²Endocrinology Department, Rabta University Hospital, Tunis, Tunisia

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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 groups¹-⁵. 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 diabetes⁶. Vitamin D
Blood samples (10 ml) were collected from 439 unrelated individuals. The exclusion criteria included fasting blood glucose levels of more than 100 mg/dl or a 2-h post-challenge glucose level ≥ 11.1 mmol/l. The aim of the present study was to analyze the association between the Fok1 polymorphism of the VDR gene and T2DM 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. 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.

Information on demography, socio-economic status, education, lifestyle and mental health was collected. Weight and height were measured. Body mass index (BMI; kg/m²) was calculated and obesity was defined as BMI ≥ 30 kg/m² for both genders. 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.

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.

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

Forward: 5’-AGCTG GCCCTGGCACTGACTCTGCTCT-3’
Reverse: 5’-ATGGAACACCTTGTTCTTCTCCCTC-3’

The PCR product was digested by the restriction enzyme Fok1 (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.
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. \( \chi^2 \) 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.

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 I.** Demographic and clinical characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>T2DM (n = 439)</th>
<th>Controls (n = 302)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>55.94 ± 9.76***</td>
<td>49.38 ± 9.63</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>30.07 ± 5.36***</td>
<td>27.88 ± 5.59</td>
</tr>
<tr>
<td>Dyslipidaemia (%)</td>
<td>34.9***</td>
<td>20.0</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>562***</td>
<td>16.3</td>
</tr>
<tr>
<td>Obesity (%)</td>
<td>43.8***</td>
<td>28.4</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>12.6**</td>
<td>21.6</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>9.76 ± 4.38***</td>
<td>4.99 ± 0.49</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.99 ± 1.03</td>
<td>4.92 ± 0.95</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/l)</td>
<td>3.19 ± 0.91**</td>
<td>2.97 ± 0.82</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.13 ± 0.44***</td>
<td>1.29 ± 0.33</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.55 ± 0.93*</td>
<td>1.40 ± 0.92</td>
</tr>
</tbody>
</table>

T2DM, type 2 diabetes mellitus; LDL, low density lipoprotein; HDL, high density lipoprotein

\( *P<0.05, **P<0.01, ***P<0.001 \) compared to controls
were controversial (Table III). Bid et al\textsuperscript{16} using FokI, BsmI, and TaqI demonstrated that there was no link between polymorphisms in the VDR gene and T2DM. Malecki et al\textsuperscript{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\textsuperscript{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 = 1.28 - 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\textsuperscript{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\textsuperscript{9}. Other explanation for the diversity of the results are selection criteria adopted for

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Authors & Population & Year & T2DM & Controls & OR (95% CI) & Significant results \\
\hline
Malecki et al\textsuperscript{15} & Polish & 2003 & 308 & 239 & 1.08 (0.85 - 1.37) & No \\
Bid et al\textsuperscript{16} & Indian & 2009 & 100 & 160 & 0.72 (0.49 - 1.06) & No \\
Al-Daghri et al\textsuperscript{17} & Saudi & 2012 & 368 & 259 & 0.80 (0.41 - 1.59) & No \\
Vedralova et al\textsuperscript{18} & Czech & 2012 & 116 & 118 & 1.12 (0.77 - 1.62) & No \\
Li et al\textsuperscript{19} & Asian & 2013 & 104 & 77 & 1.93 (1.23 - 3.04) & Yes \\
Mackawy et al\textsuperscript{28} & Egyptian & 2014 & 63 & 60 & 0.51 (0.37 - 0.72) & No \\
Errouagui et al\textsuperscript{29} & Moroccan & 2014 & 176 & 177 & 0.35 (0.14 - 0.83) & Yes \\
\hline
\end{tabular}
\caption{Associations with type 2 diabetes mellitus of the VDR gene in various sample populations}
\end{table}

N, number; T2DM, type 2 diabetes mellitus

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Genotypes & N (%) & Controls & Unadjusted odd ratios & P value \\
\hline
CC & 231 (52.6) & 168 (55.6) & 1.08 (0.85 - 1.37) & No \\
CT & 180 (41.0) & 117 (38.7) & 0.72 (0.49 - 1.06) & No \\
TT & 28 (6.4) & 17 (5.6) & 0.80 (0.41 - 1.59) & Yes \\
\hline
Alleles & & & & \\
C & 0.73 & 0.75 & 1.12 (0.77 - 1.62) & No \\
T & 0.27 & 0.25 & 1.19 (0.63 - 2.25) & No \\
\hline
\end{tabular}
\caption{Genotype distribution and allele frequencies of VDR-FokI polymorphism}
\end{table}

were controversial (Table III). Bid et al\textsuperscript{16} using FokI, BsmI, and TaqI demonstrated that there was no link between polymorphisms in the VDR gene and T2DM. Malecki et al\textsuperscript{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\textsuperscript{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 = 1.28 - 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\textsuperscript{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\textsuperscript{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[51]. 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.

Acknowledgment

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Conflicts of Interest: None.

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*Reprint requests*: Dr Riadh Jemaa, Laboratoire de Biochimie, Hôpital la Rabta, 1007, Jabbari, Tunis, Tunisia
e-mail: jemaa_riadh@yahoo.fr