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Lack of association between vitamin D receptor Taq I polymorphism and diabetic nephropathy in type 2 diabetes mellitus patients

Noha M. Sharkawy[1]*, Abdel- Rahman B. Abdel-Ghaffar[1], Doaa Mostafa Gharib[2] and Dina M. El Seoudi[2]

[1] *Biochemistry Department, Faculty of Science, Ain Shams University, Cairo, Egypt.*
[2] *Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Cairo University, Egypt.*

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ABSTRACT

The impact of several environmental and genetic factors on diabetes and its complications is well documented, however, there is an urgent need to understand more about genetic risk factors associated with this disease. Diabetic nephropathy (DN) is one of the most common complications of diabetes mellitus (DM). There's a concept suggesting that despite DM is controlled or not, some genetic factors may influence susceptibility to nephropathy. To assess the possible association of *Taq I* polymorphism of vitamin D receptor (VDR) with type 2 diabetes mellitus (T2DM) and DN in Egyptian patients taking in consideration some biochemical parameters and risk factors. A cross-sectional study enrolled 90 unrelated subjects who were divided into 3 groups: 1) Group1: included 30 healthy subjects; 2) Group 2: included 30 patients with type 2 diabetes mellitus (T2DM) without nephropathy; 3) Group 3: included 30 T2DM patients with DN. Samples were collected from the outpatient clinics of the Internal Medicine Department, El- Kasr Al-Aini hospital, Cairo University. Biochemical parameters were measured. *Taq I* polymorphism in exon 9 of vitamin D receptor (VDR) gene was analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis using *Taq I* restriction enzyme. There was no statistically significant difference in genotypic and allelic distribution of the VDR *Taq I* polymorphism between T2DM patients with DN and those without DN, in comparison to control subjects. In conclusion, data of the present study suggest that VDR *Taq I* polymorphism is not associated with the risk of T2DM or the development of DN.

Introduction

Type 2 diabetes mellitus (T2DM) is one of the major chronic and fastest growing public health problem worldwide, with increasing prevalence in the recent years [1].T2DM is the most common form of DM, which results from defects in insulin secretion from the beta cells (β-cells) and/or insulin resistance in peripheral tissues, leading to chronic hyperglycemia [2]. Diabetic nephropathy (DN) is one of the most common diabetic microvascular complications that currently ranks as the first cause of end-stage kidney disease in the world [3].The progression of kidney disease in patients with diabetes can take many years, and interventions such as glycemic control, blood pressure control, and inhibition of the renin-angiotensin-aldosterone system have been shown to slow this progression [4].

* Corresponding author.
E-mail address: Noha_sharkawy86@yahoo.com

Adverse outcomes of DM and DN can be prevented or delayed through early detection and treatment [5]. Accumulating evidence indicates that heredity is the primary contributor to the cause of the disease. More than 58 genes have been reported to be correlated with the pathogenicity of T2DM [6]. Vitamin D stimulates insulin secretion from the pancreatic β-cells directly through its interaction with the vitamin D receptor (VDR) / retinoid X receptor (RXR) heterodimer forming (1,25(OH)2D3-RXR-VDR) complex which binds to the vitamin D responsive elements found in the insulin gene promoter region to enhance the transcriptional activity of the insulin and insulin secretion[7]; It increases the sensitivity of cells to insulin indirectly through its regulatory role in the calcium flux through the cell membrane for the secretion of insulin[8]. It also improves insulin sensitivity and glucose haemostasis, directly, by its action on vitamin D receptors in both skeletal and

adipose tissue, which is peripheral insulin responsive tissue [9]. Moreover, vitamin D preserves pancreatic cell function and downregulates inflammation by the antiapoptotic action, which is mediated by downregulating Fas related pathways (Fas/Fas-L) and modulates the generation and effects of cytokines [10]. It also upregulates the expression of calbindin-D28K, which is found in many tissues including pancreatic β-cells and interferes with cytokines generation [11]. Therefore, vitamin D polymorphism may be related to impaired insulin secretion in T2DM [12].

The vitamin D receptor (VDR) gene is a candidate gene for susceptibility of several diseases [13]. There are accumulating evidence to suggest that VDR polymorphisms participated in a number of diseases including DM and DN [14]. As VDRs are widely distributed along several body tissues, their gene polymorphisms may affect the risk of vitamin D related-metabolic disorders [15].

Numerous pathways are activated during the course of DM such as increased polyol synthesis, formation of advanced glycated end products (AGEs), activation of protein kinase C (PKC) and enhanced flux through the hexoasmine pathway [16]. These pathways individually or collectively play a role in the induction and progression of DN [17]. Various studies have postulated that hyperglycemia leads to the induction of oxidative stress, a condition which increases the production of reactive oxygen species (ROS) and decreases the antioxidant defense mechanisms of the cell [18]. This imbalance results in the initiation of the previously mentioned biochemical events implicated in the pathogenesis of DN and underlying detrimental effects of hyperglycemia- induced tissue damage [19].

Taq-I polymorphism of VDR is one of the most frequently reported VDR polymorphisms that is resulted from a substitution of cytosine with thymine in exon 9 which is located near the 3′-untranslated region (3′-UTR) and is thought to reduce the mRNA stability and therefore decrease the mRNA levels of VDR gene [20]. The genetic variations in the VDR gene influence levels of VDR protein expression [21]; therefore VDR gene polymorphism may restrict vitamin D to perform its anti-inflammatory function by altering the 1, 25(OH)2 D3 binding sites [22]. Hence this study was designed to evaluate the association of the *Taq I* VDR polymorphism with DN in Egyptian patients with T2DM.

Material and methods

Subjects

Peripheral blood samples were collected from 30 T2DM without DN and 30 T2DM patients showing nephropathic complications. All patients were recruited from the outpatient clinics of the Internal Medicine Department, Kasr Al-Aini hospital (Cairo University). In addition, 30 healthy subjects were included. The patients and control groups were selected from Egyptian population and had similar medical and demographic characteristics including duration of diabetes, sex and age. Written informed consent was obtained from subjects in accordance with the principles of the Declaration of Helsinki **[23]**. The bias factors such as infections, allergic conditions and smoking were excluded from the study.

Demographic data and biochemical assessment

Demographic and clinical characteristics, including the onset of disease, age, sex and blood pressure measurements were collected and represented in **Table 1**. Blood samples were drawn from the subjects for biochemical analysis and DNA extraction. HbA1C was measured by ion exchange resin method described by (Trivelli, 1971) using a kit provided by NS Biotec (Egypt). Plasma insulin was measured using an ELISA BioVendor kit (Czech), insulin resistance by homeostatic model assessment was calculated using the following formula: (HOMA-IR = {fasting insulin [mIU/L] × fasting glucose [mmol/L]}/22.5) [24]. ß-cell function (HOMA ß %) was calculated using the formula: {20 × fasting insulin [µIU/ml] / (fasting glucose [mmol/ml]-3.5)} [24]. In addition, fasting glucose, serum urea, serum creatinine were measured using commercial kits provided by Biomed (Egypt) and microalbuminuria was measured using a kit provided by EliTech (France).

Genomic DNA extraction

To extract genomic DNA, peripheral blood was collected on EDTA and genomic DNA was extracted using Analytik Jena AG kit (Germany) according to the manufacture's guidelines. Extracted DNA was aliquoted for each sample and stored at -20°C for further analysis.

PCR amplification:

PCR amplification was performed in a total volume of 50 µl mixture containing 5 µl of template DNA,1 µl of 10 mM dNTPs, 1 µl *Taq* polymerase, 37 µl Diethyl Pyrocarbonate (DEPC) water, 5 µl 10× PCR buffer and 1 µl (50 Pmol) of each primer. The mixure was incubated for 5 min. at 95^0C for initial denaturation, followed by 30 cycles of 1 min. at 95^0C, 1 min. at annealing temperature 61^0C, 2 min. at 72^0C and additional 10 min. incubation at 72^0C for final extension. The PCR fragments (490 bp) were digested using *Taq I* restriction enzyme. The digestion mixture was incubated for 16 hr. at 65^0C. The digested samples were separated by electrophoresis on 1.5 % agarose gel stained with ethidium bromide and were visualized on a UV transilluminator (BDA digital, Biometra, Germany).

Detection of polymorphisms:

TaqI polymorphism withih exon 9 of the VDR gene was analyzed as previously described **[21]**. Briefly, primers provided by Vivantis technologies (Malaysia) were designed to flank a known *Taq*-I polymorphism carried within exon 9.

Amplicons were subjected to restriction digestion with the *Taq I* enzyme and the products were separated on an agarose gel. Alleles were scored according to the fragment patterns. Alleles digested by *Taq-I* were scored as T whereas alleles not digested by *Taq-I* were scored as t.

The sequence of primers used for polymerase chain reaction amplification of the desired region within exon 9 is as follows:

Forward Primer	5' CAG AGC ATG GAC AGG GAG CAA3'	Gene bank accession number: NM_001017536.1
Reverse Primer	5' CAC TTC GAG CAC AAG GGG CGT TAG3'	

Statistical analysis

Data analysis was done using SPSS software, version 20.0 (Statistical Package for Social Science) (SPSS Inc., Chicago, Illinois, USA). Data were expressed descriptively as mean ± standard deviation (SD) for quantitative parametric data, and as frequency & percentages for qualitative values. Genotype frequencies and allele frequencies were compared by Chi-square (χ^2) test.

Results

Table 1: Demographic and clinical parameters of diabetic patients and controls

Parameters		Control	T2DM without nephropathy	T2DM with nephropathy
Age (years)		51.53 ± 6.11	51.10 ± 6.95	52.53 ± 7.32
Sex (male/female)		M 13 / F 17	M 14 / F 16	M 13 / F 17
Duration of diabetes (years)		---	5.28 ± 2.05	8.37 ± 2.72** (versus T2DM)
Hypertension (Percentage %)	**Negative**	30 (100.0%)	11 (36.7%)	4 (13.3%)
	Positive	0 (0%)	19 (63.3%)	26 (86.7%)
HbA1C %		4.54 ± 0.61	8.55 ± 0.52**	8.66 ± 0.92**
Fasting glucose (mmol/L)		5.22 ± 0.63	9.95 ± 0.84**	10.71 ± 0.97**
Plasma insulin(mIU/L)		4.54 ± 0.61	8.55 ± 0.52**	8.66 ± 0.92**
HOMA-IR		2.02 ± 0.42	6.23 ± 1.82**	7.01 ± 1.96**
HOMA-ß%		119.12 ± 59.82	43.96 ± 11.59**	41.90 ± 14.07**
Urea (mg/dl)		52.17 ± 9.37	65.60 ± 13.64*	157.23 ± 38.48**
Creatinine (mg/dl)		1.46 ± 0.32	1.98 ± 0.97	7.36 ± 2.42**
Microalbuminuria (mg/24h)		13.29 ± 3.84	94.30 ± 19.94**	525.93 ± 160.25**

Data were shown as mean ± SD (standard deviation) and percentage (%). Significant differences were shown versus control (*$P \leq 0.05$ and 0.001) was considered significant and highly significant, respectively).

DNA extraction and genotyping

For DNA analysis, the PCR-RFLP method was used to determine the distribution of genotype and allele frequencies of the *TaqI* VDR gene polymorphism. DNA was extracted using InnuPREP blood DNA extraction Analytic Jena AG kit (Germany), and PCR amplification was performed as shown in **Fig. 1**.

Polymorphism within the VDR was scored according to PCR-RFLP of exon 9. An example of a typical *Taq-I* digestion of exon 9 and how it is scored is shown in **Fig. 2**.

Evaluation of the polymorphism within exon 9 of the VDR gene by *Taq-I* restriction digestion showed that the prevalence of T/T genotype was 7 (23.3 %) in controls, 3 (10 %) in T2DM patients without nephropathic complications and 4 (13.3 %) in T2DM patients showing nephropathic complications **Table 2**. Our results also revealed that the frequency of T/t genotype was 10 (33.3 %), 19 (63.3 %) and 17 (56.7 %) in control, T2DM patients without nephropathy and T2DM patients with nephropathy, respectively **Table 2**. The value for the t/t genotype in control was 13 (43.3 %), T2DM without nephropathy was 8 (26.7 %) and T2DM patients showing nephropathy was 9 (30 %) as shown in **Table 2**.Our results demonstrate that the genotype differences were non-significant among different groups ($P < 0.187$).The difference among groups regarding the frequency of T and t alleles was also not significant **Table 2**.

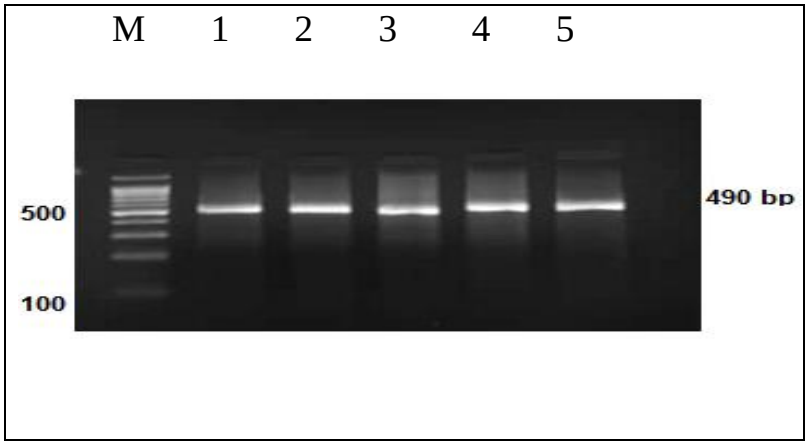

Fig. 1: The amplification of exon 9 of VDR gene [**M**: **DNA ladder marker 100 bp**]. Lanes 1and 5 denote DN group patients. Lane 2 and 3 denote T2DM group and lane 4 denotes control.

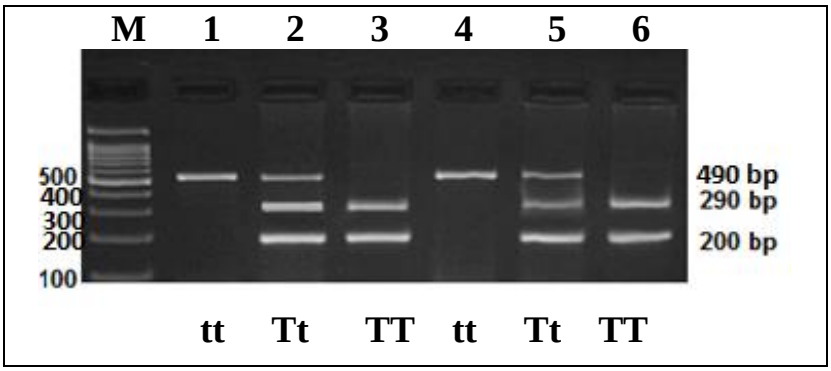

Fig. 2: The digested products of exon 9 of VDR gene. [**M: DNA ladder marker 100 bp**]. Lanes 1 and 4 denote mutant homozygote tt (490 bp). Lanes 3 and 6 denote wild homozygote TT (200 bp, 290 bp). Lanes 2 and 5 are mutant heterozygote Tt (200bp, 290bp and 490 bp). Where lanes 1 and 4 denote DN group patients, lanes 2, 5 and 6 denote T2DM group and lane 3 denotes control.

Table 2: The genotype frequencies of the *Taq- I* polymorphism and allelic distribution in the control versus patient groups

Genotype	**Condition**			***P*-Value**
	Control (n=30)	**T2DM patients without nephropathy (n=30)**	**T2DM patients with nephropathy (n=30)**	
T/T (%)	**7 (23.3 %)**	**3 (10 %)**	**4 (13.3 %)**	**0.187**
T/t (%)	**10 (33.3 %)**	**19 (63.3 %)**	**17 (56.7 %)**	
t/t (%)	**13 (43.3 %)**	**8 (26.7 %)**	**9 (30 %)**	
Alleles				
T (%)	**24 (40 %)**	**25 (41.7 %)**	**25 (41.7 %)**	**0.977**
t (%)	**36 (60 %)**	**35 (58.3 %)**	**35 (58.3 %)**	

Discussion

T2DM and its complications impose a tremendous burden both on patients and healthcare systems; therefore the prevention and control of T2DM is a tough task for healthcare providers and relevant scientists **[14]**.DN is one of the most common diabetic microvascular complications that currently rank as the first cause of end-stage kidney disease in the world, as DN shortens the life expectancy of patients if untreated. Therefore, its prevention or even early diagnosis and treatment is important [3, 25].

Many studies have indicated strong involvement of genetic factors in diseases including DM [19].Therefore, screening genes to find genetic variants and evaluating their combined and additive effects on the risk of T2DM and insulin function could help identify individuals who are at risk and ultimately lead to new therapies for the prevention and treatment of T2DM [20]. The VDR gene is a candidate gene for susceptibility of several diseases, including DM and DN [14].

In this study, the patient and control groups were matched for duration of diabetes, sex, age and socioeconomical status. The present study indicated non-significant difference between *Taq-I* evaluated genotypes of exon 9 within the VDR gene between both diabetic groups and controls. This was in agreement with **Bid *et al.*** [26], who demonstrated that there was no link between *Taq I* polymorphism in the VDR gene and type 2 diabetes and **Dilmec *et al.*** [27], who failed to find any relationship between this polymorphism and T2DM patients in Turkish population. According to the data which support the view that *Taq I* VDR polymorphism has no association with diabetes, this may be explained as *Taq I* polymorphism is a silent substitution which does not account for an amino acid change in VDR protein and therefore is not expected to alter VDR structure and function [28].

Moreover, conflicting results were found regarding this correlation, as some studies showed that the distributions were different between patients with diabetes and control groups. For instance, the studies by [**Nosratabadi** [29]; **Aldaghri *et al.*** [30]; **Taneja *et al.*** [28] and **Malik *et al.*** [15]] observed that *Taq I* polymorphism is significantly associated with T2DM. The apparent discrepancies between this study and others could be a result of the effect of ethnic differences related to the distribution of *Taq I* VDR polymorphism in these populations [28].

Nostrabadi *et al.* [31] examined the possible correlation between the *Taq I* polymorphism and VDR gene expression and found that the presence of the wild type TT genotype of *Taq I* polymorphism is known to be associated with high copy number of VDR mRNA expression. Hence, it may be hypothesized that the patients lacking wild genotype may have low copies of VDR gene transcripts which may probably affect level of VDR protein, and hence, the glucose metabolism too [31]. On the other hand, the results of the current study showed that TT genotype was detected in only 10 % and 13.3 % in DM and DN groups, respectively, in comparison to controls (23.3 %). However, this decrease was not significant among groups (P= 0.187).

The mutant genotype (tt) percentages appeared to be lower in both diabetic groups DM and DN (26.73 % and 30 %, respectively), compared to controls (43.3 %). Also, in both patient groups, the number of patients with the heterozygous Tt genotype were shifted to be higher 63.3 % and 56.7 % in DM and DN, respectively, compared to controls (33.3 %). However, these differences were also non-significant.

The current study showed that the values for the T allele frequency of DM and DN patient groups were equally distributed (41.7 %), and similar to the control group (40 %), while the values for the t allele frequency in DM and DN patients were (58.3 %), and in the control group was (60 %). In addition, the allele differences were not significant in DM and DN groups in comparison to the control group (p= 0.977). This result was consistent with the previously reported study by **Nostrabadi *et al.***[31], who demonstrated a non-significant difference within *Taq1* evaluated alleles in T2DM in Southern Iranian patients.

Taneja *et al.* [28] showed that there is a link between heterozygous genotype (Tt) and wild genotype (TT) of *Taq I* polymorphism and T2DM in Caucasian population. The association of genotypes of *Taq I* polymorphism with T2DM may be explained by that *Taq I* is localized near the 3′-untranslated region (3′-UTR) of this gene; so may affect mRNA quality and stability by interfering with VDR mRNA expression, subsequently affecting VDR protein level and/or activity [32, 33].

Association of exon 9 VDR polymorphism with nephropathy complication also were evaluated by researchers. For example, **Maia *et al.*** [34] and **Bucan *et al.*** [35] did not find a significant association between *Taq I* VDR polymorphism and DN risk.

This finding was in agreement with the results of the present study, which supports the point of view that there was no significant difference between groups in relation to exon 9 polymorphism and DN risk. On the contrary, **Yin *et al.*** [36] reported that Tt genotype of *Taq I* variant might be risk factors for DN progression via vitamin D-VDR signaling in podocytes which may play a critical role in the protection against DN, as VDR activation could decrease inflammatory responses of intrinsic renal cells in DN.

Based on the current study, and results from previous works, it can be concluded that exon 9 VDR polymorphism is neither associated with T2DM nor nephropathic complications in this type of diabetes because non-nephropathic patients also showed the same polymorphism. The role of VDR polymorphisms in T2DM and its nephropathy should be evaluated in a much larger population by other researchers, simultaneously to increase the scope and power of the study.

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