Association of Vitamin D Receptor Ggene Ppolymorphisms and Type 1 diabetes in Egyptian Population.

ارتباط التباين الجيني لمستقبلات فيتامين دي مع مرض السكري النوع الاول في المجتمع المصري

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Abstract

The human vitamin D receptor (VDR) gene is located on chromosome 12q12–q14, and four common nucleotide polymorphisms have been identified. Several studies have found a relationship between polymorphisms of the (VDR) gene and development of type 1 diabetes (T1DM). The association of VDR polymorphisms and susceptibility to T1DM in the Egyptian population were examined in 60 individuals with type 1 diabetes and compared with healthy 60 persons. Single nucleotide polymorphisms (SNP) genotyping was performed using PCR and *BsmI* and *FokI*, by using two techniques, allele specific PCR technique and restriction fragment length polymorphism – PCR (RFLP-PCR). Data were analyzed using the chi square. The result approved that the genotype TA in SNP *FokI* was risk factor among type 1 diabetes mellitus patients combination which conferred strongest susceptibility to T1DM (P=0.004) while the SNP *BsmI* did not showed any significance between cases as compared with control (P=0.493). The results of the current study indicated that VDR polymorphisms are associated with increased risk of T1DM in the Egyptian population. The difference in the association of the aforementioned SNPs variants with T1DM among different populations may be attributed to the presence of multiple susceptibility alleles.

Keywords: Vitamin D receptor, Genotype, Type 1 diabetes, Genetic susceptibility, SNP, *BsmI, FokI*

الملخص

يقع جين فيتامين دي المستقبل على الكروموسوم 12(q14–q12), وهنالك اربع مواقع شائعة للنيكلوتيده متغيره الشكل لها علاقة بمرض السكري من النوع الاول (T1DM) تم تسجيلها من خلال الدراسات السابقه. تم التحري عن الارتباط بين بين التباين الجيني لمستقبلات الفيتامين دي وارتباطه بمرض السكري من النوع الاول, وتم فحص عينة مكونة 60 شخص مصاب بمرض السكري من النوع الاول مقارنة مع 60 شخص كمجموعه سيطره في المجتمع المصري غير مصابين بمرض السكري. تم التحري عن التباين الجيني في المواقع *BsmI* and *FokI* باستخدام PCR-RFLP و allele specific. حللت البيانات احصائيا باستخدام اختبار مربع كاي. اظهرت النتائج ان التركيب الوراثي TA في النيكولتيده متغيره الشكل في مواقع القطع *FokI* تعد كعامل خطر للاصابة بمرض السكري من النوع الاول وفقا للدلالات الاحصائية (P=0.004), بينما النيكولتيده المتغيره الشكل *BsmI* لم تظهر اي ارتباط على مستوى المرضى والمجموعه الضابطه مع مرض السكري من النوع الاول وفقا للدلالات الاحصائية (P=0.493). اثبتت هذه النتائج ان هنالك ارتباطا مع ارتفاع خطر الاصابة بمرض السكري من النوع الاول والتباين الجيني لمستقبلات فيتامين دي في المجتمع المصري, ويعزى هذا الاختلافات في النيكلوتيدات وجود اكثر من اليل للنيكلوتيده متغيره الشكل في المواقع السالفه الذكر على مستوى اختلاف المجتمعات.

الكلمات المفتاحية: فيتامين دي المستقبل، التركيب الوراثي، السكري من النوع الاول، القابلية الوراثية

Introduction

Type 1 diabetes mellitus (T1DM) results from β-cell destruction, usually leading to absolute insulin deficiency. Testing for islet-cell antibodies (ICA) or other autoantibodies (antibodies to glutamic acid decarboxylase [anti-GAD], insulin, and to the tyrosine phosphatase IA-2) in serum may be helpful if establishing the diagnosis is important; a positive result is indicative of immune-mediated or type 1A diabetes [1]. Relationship between the vitamin D receptor (VDR) gene and T1DM has been noted in several populations [2-4] has been recognized for its immunomodulatory effects [5], and action on insulin secretion [6-9]. This molecule has been recognized for its acts through activation of the (VDR) that is encoded by a gene located in chromosome 12q12 [10]. Several polymorphisms have been described for the VDR gene [11]. Most studied are *ApaI, BsmI, FokI,* and *TaqI*. The VDR *FokI* polymorphism in exon 2 leads to an

alternative transcription initiation site, resulting in a VDR protein with addition of three amino acids [12, 13]. The VDR *BsmI* polymorphism is located in the intron between exon 8 and 9 and has no established functional role as of yet [13].

The aim of this study was to determine the association of T1DM with the two SNPs (SNP T/A rs#2228570 & SNP G/A rs#1544410) in - vitamin D receptor to the susceptibility to type 1 diabetes in the Egyptian population.

Materials and methods

Study populations

A total of 60 type 1 diabetic patients (25 males /35 females) mean age ±SD 11.2±3.7, 60 healthy individuals (33 males / 27 females) mean age ±SD 27.2± 6.4, family history(25 positive/ 35 negative to family history, disease onset (years) mean±SD 5.3±3.5, were enrolled in this study and recruited at the El-Shatby University Hospital, Faculty of Medicine Alexandria – University, Egypt. Patients diagnosed according to WHO criteria [14]. Patients had been diagnosed on the basis of classical clinical presentation, first-degree family history of diabetes, history of chronic diabetes complications, and treatment of diabetes. Healthy controls had no personal or first-degree history of diabetes and were free from T1DM. The Ethics Committees of participating universities and university hospitals approved the study, and informed consent was obtained from all participants.

Blood sampling was carried out, one ml of venous blood sample was collected in EDTA tubes from each individual (patient or healthy control) and was stored as whole blood at -20^{o}C for subsequent DNA isolation. Genomic DNA was isolated from whole blood according to Sambrook *et al* (1989) [15].

Genotyping of vitamin D-receptor gene polymorphism

Two SNPs (SNP T/A rs#2228570 & SNP G/A rs#1544410) in vitamin D-receptor were genotyped among the participants group in this study.

In vitamin D-receptor gene was genotyped among the participants groups in this study. The vitamin D-receptor SNPs (SNP T / A rs#2228570 & SNP G / A rs#1544410) was amplified by polymerase chain reaction (PCR) using allele specific PCR technique and restriction fragment length polymorphism –PCR (RFLP-PCR) as shown in Table 1. Four primers for SNP (SNP T / A rs#2228570) (two allele specific primers, forward, control and common reverse primer) were designed based on the nucleotide sequence of a partial fragment (retrieved from the online dbSNP) of the gene containing the target SNP. The PCR product in SNP G/A rs#1544410 was digested using an appropriate restriction enzyme *HhaI*, The recognition site of *HhaI* is -GCGC- after using forward primer 5-CCTCACTGCCCTTAGCTCTG-3 and reverse primer 5-TCTCACCTCTAACCAGCGGA-3as shown in table 1 to amplify the SNP G/A rs#1544410, the target SNP localized in the nucleotide sequence representing the recognition site of this restriction enzyme. The polymorphism was visualized by separating the DNA fragments in a 2% agarose gel that was stained with ethidium bromide and illuminated by UV. To validate the PCR- allele specific and restriction fragment length polymorphism results as showed in figure 1 and figure 2, all primers used in this study were newly designed using Primer Blast online programmed (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

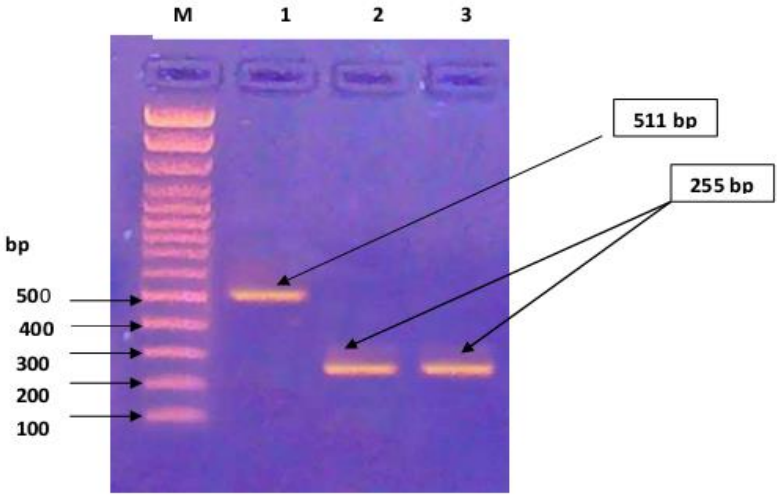

Fig. (1): Shows 2% agarose gel electrophoresis for allele specific PCR for Vit.D SNP T>A rs#2228570 (*Fok1*). M: 100 bp DNA ladder from GeneDire*X*®. Lane1: PCR product upon using controls forward primer, Lanes 2 and 3: PCR products upon using allele specific T primer and allele specific A primer, respectively. Heterozygous genotype will give positive reaction upon using both allele specific primers. However, homozygous genotype will give positive reaction upon using only one of these allele specific primers.

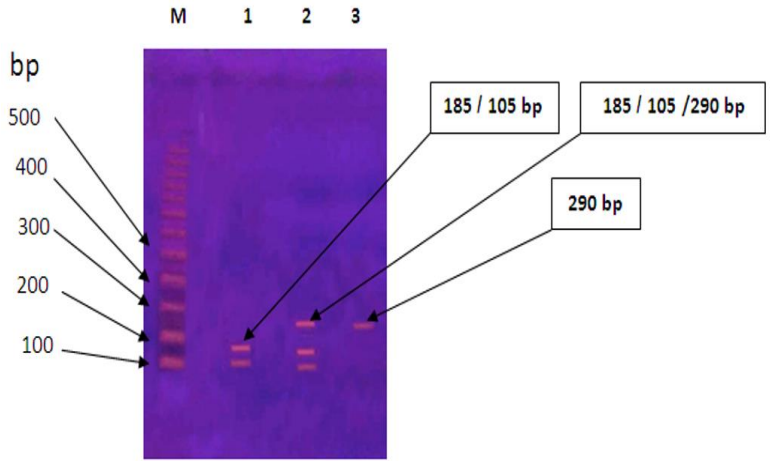

Fig.(2): Shows 2% agarose gel electrophoresis for RFLP- PCR for *VitD Receptor* SNP G>A (rs#1544410). M: 100 bp DNA ladder from GeneDire*X*®. Lane 1 represents a homozygous GG genotype as the G allele PCR products cleaved by FastDigest® *HhaI* into 104 bp and 185 bp. Lane 2 represents a heterozygous GA genotype as G allele PCR products cleaved by FastDigest® *HhaI* into 104 bp and 185 bp while A allele PCR products were still 290 bp. Lanes 3 represents a homozygous AA genotype after cleavage by FastDigest® *HhaI* still 290 bp.

Statistical analysis of data

Statistical analysis of the data was done to correlate genotype distribution and allele frequencies using SPSS package version 11. The frequencies of alleles, genotypes in different groups were compared using the chi-squared test (X2), T-test and Mann Whitney test were used to test the significance of results of quantitative variables. Odds ratio and 95% confidence interval (95% CIs) were calculated for different studied parameters. The confidence interval (CI) at 95% was used to describe the amount of uncertainty associated with the samples [16, 17]. A 95% confidence level means that 95% of the intervals would include the parameter. The significance of the results was taken at the $P \leq 0.05$ level of significance.

Table (1): Primers sequences, PCR conditions, length of PCR products, restriction digestions and products of *restriction digestion*

SNPs	Primers sequences	PCR Conditions	Restriction Digestion	Size of PCR roducts digestion products
VitD Receptor Fok1 **SNP T/A (rs#2228570) ****	**T-allele specific primer: F1: 5-GCTTGCTGTTCTTACAGGGA T-3 A-allele specific primer: F2:5-GCTTGCTGTTCTTACAGGGA A-3 Forward control primer: 5-CCTGACAGATGCAACATCTGAAAC-3 Common reverse primer: 5-GATGTGAAAAATGCAAGGGCTC-3**	**An initial denaturation at 95°C for 5 min -Then, 30 cycles each cycle consisted of denaturation at 94°C for 60s, annealing at 50 °C for 30s and extension at 72°C for 30 s. -A final extension at 72°C for 10min.**	**-**	**Allele T: 255 bp Allele A: 255 bp** **Control fragment: 499 bp**
VitD Receptor BsmI **SNP G/A (rs#1544410)* ***	**Forward primer: 5-CCTCACTGCCCTTAGCTCTG-3 Reverse primer: 5- TCTCACCTCTAACCAGCGGA-3**	**An initial denaturation at 95°C for 5 min -Then, 30 cycles each cycle consisted of denaturation at 94°C for 60s, annealing at 50 °C for 30s and extension at 72°C for 30 s. -A final extension at 72°C for 10min.**	**PCR product was digested with HhaI Fast digest enzyme . Total volume of digestion reaction mixture (30 µl) contained: 20µl of PCR product, 1 µl of HhaI (Fast digest) enzyme, 3 µl of 10X buffer, 1 µl of BSA and 5 µl nuclease free water. This mixture incubated at 37°C for 20 minutes.**	**PCR product: 290 bp** **Restriction digestion products : 104 bp and 185 bp Allele G: 104 bp and 185 bp Allele A: 290bp**

Results and Discussion

This study demonstrates that VDR gene polymorphisms are associated with an increased risk of T1DM in Egyptian population which would be explained by differences in BsmI and Fok1 genotype distribution between T1DM patients and control group The present results revealed that the allele and genotypic distributions of GG, AA and GA were convergent, also did not showed significance according to the statistical analysis for the SNP BsmI G/A (rs#1544410). On the other hand, there was a statistical significant difference between the two groups (P=0.004*) of the SNP Fok1 T/A (rs#2228570). The results showed that TA genotype distributions greater than TT and AA genotype according to the statistical analysis and this consider is a risk factor among T1DM patients as shown in Table (2).

Table (2): Vitamin D-receptor gene polymorphism and allele frequencies among diabetic patients and their control

Gene polymorphism	Cases		Control		Significance	OR (95% CI)
	No.	%	No.	%		
BsmI G/A						
GG	**8**	**13.3**	**4**	**7.1**	**X^2=1.414**	**--**
AA	**13**	**21.7**	**11**	**19.6**	**P=0.493**	**0.6 (0.1-3.1)**
GA	**39**	**65.0**	**41**	**73.2**		**0.5 (0.1-1.9)**
allele frequencies						
G	**60**	**0.24**	**60**	**0.17**		**----------**
A		**0.76**		**0.83**		
Fok1 T/A						**95% CI for**
TT	**16**	**26.7**	**7**	**11.7**	**X^2=33.700**	**difference:**
AA	**21**	**35.0**	**12**	**20.0**	**P=0.004***	**(-46.473961;**
AT	**23**	**38.3**	**41**	**68.3**		**46.473961)**
allele frequencies						
A		**65**		**54.1**		**----------**
			60			
T		**55**		**45.9**		

X^2: Chi-Square test ***significant at P≤0.05** **--NA not applicable**

In previous study Lemos *et al*.(2008) [18] suggested that the single nucleotide polymorphisms of the *VDR* gene are unlikely to contribute significantly to T1DM susceptibility in both loci in the Portuguese population. Nejentsev, *et al*. (2002) [19] indicated that common sequence variation in the *VDR* gene has no major effect in T1DM in both loci in the U.K populations. Otherwise Ban *et al*.[4] found that there is significantly higher prevalence in the *VDR-FokI* T≥ C (rs#10735810) genotype, the F allele / the FF genotype in the patients compared to the controls. They suggested that the *VDR* initiation codon polymorphism influences genetic susceptibility to T1DM among the Japanese. On the other hand, Wang *et al*.(2012)[20] suggested that FokI polymorphism in the *VDR gene* is not associated with T1DM especially in East Asians population, while *Bsm I* polymorphism in the *VDR* gene was associated with T1DM risk, especially in East Asians population. The apparent discrepancies between this study and other studies could be a result of the effect of ethnic differences related to the distribution of *VDR* polymorphisms in these populations, as well as to interactions with other genetic or environmental factors involved in the pathogenesis of type 1 diabetes mellitus.

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