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CLINICAL AND GENETIC VARIATION IN ASSOCIATION BETWEEN TYPE I DIABETES AND VITAMIN D DEFICIENCY

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ABSTRACT Diabetes is a serious concern throughout the world and various metabolic conditions are under investigation to get a clear picture on its prognosis. The aim of our study was to investigate three polymorphic sites *ApaI, BsmI* and *TaqI* of vitamin D receptor (VDR) gene on chromosome 12q (12–12q14) as candidate for type 1 diabetes mellitus (T1DM) in Saudi population. A sample of 100 Saudi classified as healthy (control,N= 50 (without T1DM) or with T1DM (N= 50) was investigated the relationship of VDR gene polymorphisms at three restriction sites *Apa*I, *Bsm*I and *Taq*I to the risk of T1DM .Blood samples were taken and DNA was extracted from whole blood. The target part of VDR gene was isolated and amplified by the polymerase chain reaction (PCR). PCR products were digested by restriction enzymes: *ApaI, TaqI, and BsmI* and electrophoresed on agarose gel. Fasting Blood Glucose (FBG) levels were statistically higher in T1DM than controls (P-value= 5.73E-12*). The difference in Vitamin D Total (Vit.D) values between each two shows non-significante difference p-value =0.5226. However, we observed that was distribution of genotypes frequency of the *BsmI* VDR gene polymorphisms differed highly significantly between T1DM and control groups (p-value =0.0081). On the other hand, there was no significant difference in genotypes and alleles frequencies of the VDR gene polymorphisms at position *TaqI* and *Apa*I between T1DM patients and control groups in Saudi population. This shows that although clinically there is no significant difference in vitamin D levels between control groups and diabetic patients, there is a significant variation at the genomic level indicating that a pharmacogenomic approach is the need of the hour to elucidate the mechanism of prognosis of metabolic diseases like diabetes.

KEYWORDS : Type 1 diabetes Melitus(T1DM);Vitamin (D) receptor (VDR) ;Gene polymorphisms ;Saudi population .

Introduction

Diabetes mellitus (DM) is a group of metabolic disorders characterized by a chronic hyperglycemic condition resulting from defects in insulin secretion, insulin action or both. T1DM is characterized by the destruction of pancreatic beta cells resulting in absence of insulin secretion, thus requiring exogenous insulin for survival (1)**.**It however is believed to involve a combination of genetic and environmental factors. It is also the most common form of diabetes in childhood and young adults' worldwide (2)**.**

The active form of vitamin D 1, 25 $(OH)_2D_3$, influences insulin secretion and is an important immune modulator (3)**,** (4) .Therefore, beta-cell damage by cytokines and other inflammatory agents might play an important role in the pathogenesis of T1DM (5)**.**

It also plays an important role in calcium homeostasis by regulation of bone cell growth, differentiation, intestinal calcium absorption and parathyroid hormone (PTH) secretion (6).

Vitamin D_3 acts through its specific receptor, a nuclear protein called vitamin D receptor (VDR). VDRs are present in pancreatic β-cells and vitamin D is essential for normal insulin secretion (7).The VDR gene is located on chromosome 12q (12–12q14) and is highly polymorphic (8).This gene includes eight protein-coding exons (exons 2–9) and six untranslated exons (exons 1a–1f) which are alternatively spliced. Four common single nucleotide polymorphisms (SNPs) including: *FokI*, *BsmI*, *ApaI* and *TaqI* which are located at the 3′ end of the VDR gene have been investigated extensively (9)**;** (10). Activation of T-cell is suppressed by VDR, thus polymorphisms in this receptor gene are associated with T-cell mediated autoimmune diseases (11).

Association between VDR gene polymorphisms and T1DM have been investigated that found to influence susceptibility to T1DM in several population (11)**;** (12).Several studies have suggested association between one or more of these SNPs and T1DM **(** (13)**;** (14)**),**but others have failed to confirm this finding (15).This inconsistency was attributed to the environmental factors that potentially interfere with the VDR genotypes (16)**.** Besides the fact that the molecular evidence for the putative relation between VDR polymorphism and such disorders remains unclear, It has been demonstrated that a length of the VDR, affected by the presence of the polymorphisms, could lead to a lower activation of target cells, since a longer VDR protein appears to have decreased transcriptional activity (17)**,** (15).Therefore, we have investigated three polymorphic sites *ApaI,BsmI* and *TaqI* of VDR gene on chromosome 12q (12–12q14) as candidate for T1DM susceptibility locus for the first time in Saudi population.

Material and Methods

Subjects

The VDR gene polymorphisms analysis study included a total of 100 Saudi volunteers of age ≤30 years. The diagnosis criteria of T1DM were according to the World Health Organization (WHO).Subjects were divided in two groups T1DM and non-T1DM. Total number of 50 Saudi patients (20 men and 30 women; mean age= 23.44±5.38 years old) with T1DM. Fifty healthy control subjects were studied (25 men and 25women; mean age =25.37±4.07 years old). They consisted of individuals with no history of diabetes or other autoimmune disease. This criterion depends upon two tests: Fasting blood glucose test (FBG) and Vitamin D Total Test **(Vit.D) .** We recorded the clinical parameters for each T1DM volunteer upon diagnosis :age ,FBG test ,Vit.D test .These medical details were obtained from patient hospital files after obtaining the consent from the administration. The same parameters were recorded for the control volunteer. All subjects were selected from those who routinely attended diabetic clinic, Association of Diabetic Patient Friends Jeddah, King Abdul Aziz University Hospital (KAUH), Jeddah, Saudi Arabia. The study was approved by the ethical committee. All participants in the study filled a questionnaire and also signed a consent form.

Study Design

Genomic DNA was extracted from whole blood samples, in bio safety cabinet, using QIAamp DNA Blood Mini Kit (QIAGEN, USA, Cat.no.51104). The extracted DNA was stored at -20˚C for PCR amplification.

For Polymerase Chain Reaction (PCR), the reactions were prepared using Maxima Hot Start Green PCR Master Mix (2x). The primers were from Biolegio, Nijmegen, Netherlands. The forward primer was (5'-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3') and the reverse one was (5'-GCA ACT CCT CAT GGC TGA GGT CTC-3'). For polymerase Chain Reaction (PCR), the master mix from Thermo Scientificwas used. The mixture (50µl) contained 2X reaction buffer, 4mMMg+2 Mg^{+2}, 4µM deoxyribonucleoside triphosphates, 0.2µM of each primer, 0.45 U *Taq* DNA polymerase and 10-30 ng of DNA

template. The total reaction volume was made up to 50µl with nuclease free water.

The PCR tubes were transferred to thermal cycle. The amplification conditions were ; an initial denaturation for 4 min at 95˚C , 30 cycles each of which consisted of (denaturation at 95 ˚C for 30 S, annealing at 60 ˚C for 1 min and an extension at 68 ˚C for 2 min), and final extension for 5 min at 72˚C and ended at hold at 4˚ C. The PCR products were verified by 1% agrose gel electrophoresis at 100 V for 30 min .Purification of Purification was done for the PCR product using Gene JET PCR Purification Kit from Thermo scientific.

VDR genotyping

Amplified PCR products (5 µl) were digested with 3000 U of each restriction enzyme from Thermo Scientific. These enzymes are *Acetobacter pasteurianus ApaI*, *Bacillus stearothermophilus BsmI* and *Thermus aquaticus YTI TaqI*. These enzymes were used to the supplier-recommended protocols by Thermo Scientific. By using the thermal cycler, the reaction was incubated in 37˚C for 1 hour which is the activation temperature of *BsmI* and *ApaI*, then the enzymes were inactivated by incubation at 65˚C for 20 min. After that, the reaction was incubated 65˚C for 1 hour which is activation temperature of *TaqI*, and then the enzymes were inactivated by incubation at 80˚C for 20 min.

Statistical analysis

All statistical analyses were performed using the MegStat® version 9.0 computer program. Descriptive data were given as mean ± standard deviation (SD).Differences among groups were tested using the t-test. Differences in distribution of the genotypes between the females and males according to T1DM and Non-T1DM were examined with the chi-square analysis. Differences in genotype frequencies were considered statistically significant for p-value < 0.05.

Results

The Main Characteristic of the study group

The volunteers in this study were classified according to Fasting Blood Glucose (FBG) test as two groups T1DM (30 Females, 20 Males) and non-T1DM (25 Females, 25 Males) totally 50 subjects each matched in age. The clinical and biochemical parameters for the two groups T1DM and control sample are shown in Table 1. The age between T1DM and non-T1DM shows significances different p-value= 0.0442< 0.05, but non-significant between females and males in two groups. The Fasting Blood Glucose (FBG) values between T1DM and non-T1DM shows significant difference p-value= 5.73E-12*
<0.05, but non-significant difference between females and males in two groups. The vitamin D Total (Vit.D) values between each two groups females and males are non- significant difference p-value =0.5226>0.05 between T1DM and non-T1DM.

Table 1 Clinical and biochemical characteristics of non-T1DM and T1DM

Parameters	T1DM Mean ± SD	Non-T1DM Mean ± SD	P-value
Age (years)	5.38±23.44	4.07±25.37	0.0442
Vitamin D Total (Vit.D) (nmol/L)	22.30±39.66	18.62±37.04	0.5226
Fasting Blood Glucose (FBG) (mmol/L)	6.023±12.024	0.451±5.408	5.73E-12*

*Highly significant difference.
Data is represented as mean ± **SD**
P-Value for control groups and T1DM.

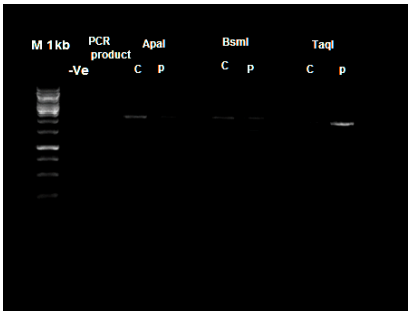

Figure 1 Digestion results of the 3 polymorphism sites. Lane 1 :1kb DNA ladder .Lane 2 :negative control, lane 3 :PCR Products (2229 bp). *ApaI* digestion:control(2229bp,1900bp,500bp)patient (2229bp,1900bp,500bp).*BsmI* digestion:control(2229bp),patient (2229bp, 1900bp, 700bp).*TaqI* digestion :control(2229bp) ,patient (2229bp,250bp).

The PCR products in our samples from Saudi volunteers, ~ 2229 bp as shown in Figure 1, were digested with the *BsmI ,TaqI and ApaI* restriction enzymes.

Statistical analysis of Genotypes Frequencies of *BsmI,TaqI* and *ApaI* Polymorphisms in study Groups at T1DM and Control samples

Frequencies of VDR alleles and genotypes in the two groups, irrespective of whether it was at females and males, shown in Table 2 respectively. Accordingly, the distribution of genotypes frequency of the *BsmI* VDR polymorphisms differed significantly high between T1DM and controls (p-value=0.0081<0.05),Bb genotypes occurring more frequently in the controls than patients. Furthermore, the frequency of B allele was also more frequent in control group compared with T1DM patients and significant difference was observed between them p-value=0.0010 <0.05.Also, there was highly significant different between females and males in control groups (p-value=0.0002<0.05). There was no significant difference in genotypes and alleles frequencies of the VDR gene polymorphisms at positions *TaqI* and *ApaI* between T1DM patients and control groups.

Table2 Distribution of VDR gene polymorphisms in patients with T1DM and non-T1DM

VDR polymorphisms	Case (n=50)		Controls (n=50)		P-value
	NO.	%	NO.	%	
BsmI Genotypes					
BB	3	6%	3	6%	****0.0081
Bb	12	24%	18	36	
bb	35	70%	29	58	
ApaI Genotypes					****0.4177
AA	6	12	3	6	
Aa	23	46	14	28	
aa	21	42	33	66	
TaqI Genotypes					**0.1326
TT	48	96	50	100	
Tt	2	4	0	0	
tt	0	0	0	0	
Bsm I Alleles					***0.0010
B	18	18	24	24	
b	82	82	76	76	
ApaI Alleles					***0.3870
A	35	35	20	20	
a	65	65	80	80	
TaqI Alleles					***0.0826
T	98	98	100	100	
t	2	2	0	0	

Discussion

This study has paved the way for a new approach to elucidating the genetic mechanism of diseases such as diabetes. In our study, clinically there was no significant difference between vitamin D levels of diabetic and non diabetic patients. This is a dubious situation, wherein it clearly shows that if we limit our analysis to clinical parameters we tend to miss out the major underlying genetic cause. Our study at the genomic level clearly indicated that varoiations of the vitamin D receptor gene polymorphisms are significantly associated with onset of diabetes. Several studies reported association between four VDR gene polymorphisms, at position *ApaI,BsmI,FokI* and *TaqI* and T1DM were inconsistent among different studies **(** (17)**,** (20)**;** (21)**;** (22)**;** (15) (23)). Due to these reasons, this study has been chosens to investigate the association of T1DM in a sample of Saudi population with VDR *Bsm-Apa-Taq* I polymorphisms and measured biochemical tests such as (FBG) levels and total vitamin D as a target for this study. Vitamin D deficiency can result from inadequate exposure to sun, in adequate alimentary intake, decreased absorption, abnormal metabolism, or vitamin D resistance (23)**.**
Recently, many chronic diseases such as cancer **(**(24)**;** (25)**)** Osteoporosis **(** (26)**;** (27)and several autoimmune diseases **(** (28)**;** (29)**)** have been linked to vitamin D deficiency. In addition, whether

consumed or produced, both forms of vitamin D(D2 and D3) are metabolized by the liver to 25 (OH) D, and then converted in the liver or kidney into 1,25-dihydroxyvitamin D (30) **.**Vitamin D metabolites are bound to a carrier protein in the plasma and distributed throughout the body . The most reliable clinical indicator of vitamin D status is 25 (OH) D because serum and plasma 25(OH)D levels reflect the body's storage levels of vitamin D, and 25 (OH) D correlates with the clinical symptoms of vitamin D deficiency (31)**.**Furthermore, vitamin D deficiency was highly prevalent in Saudi population .They cover their body completely except their faces. Wearing concealing clothing and restriction of outdoor activities has been reported previously as a risk factor for vitamin D deficiency in Female Saudi Arabia adolescents (32)**.**

We examined the association of VDR gene polymorphisms at three position (*ApaI*, *BsmI* and *TaqI*) with T1DM. Our results demonstrated alleles of *BsmI* gene polymorphisms was more frequent in healthy controls compared with T1DM patients and there was highly significant difference in allele frequency between cases and controls (P= 0.0010<0.05). Therefore, allele b in *BsmI* region is risk allele. In contrast, there is no significant difference between T1DM and controls in the allele and genotype frequency in *TaqI* and *ApaI* gene polymorphisms in Saudi population.

This outcome matches the outcome of the largest meta-analysis which found relationship between *BsmI* polymorphisms in VDR and T1DM, whereas the *FokI, ApaI* and *TaqI* polymorphisms do not appear to have a significant association with overall T1DM risk. Therefore, the *BsmI* variant B allele (BB or Bb) carriers might have a 30 % increased risk of the T1DM when compared with the bb homozygote carries (33) **.** In contrast, a meta-analysis in 2006 showed that there was no association between VDR gene polymorphisms and T1DM in case-control and family transmission studies (34).

Furthermore, the apparent discrepancies between this study and other could be a result of the effect of ethnic differences related to the distribution of VDR polymorphisms in these population as well as interactions with other genetic or environmental factors involved in the pathogenesis of T1DM (14). Therefore, the environmental factor influence levels of active vitamin D in humans are complex and a significant difference exists between vitamin D functions and VDR polymorphisms (35).

From the previous studies, it is evident that VDR gene polymorphisms to vary with different populations in their association with T1DM. There are many reports revealed their association with many physiologic phenotypes, though inconsistently (36)**.**The thing that we cannot ignore it in our result is the absence of *TaqI* genotype tt in our result. We confirmed this absent in our sample by choosing stratified random samples from different genotypes with homozygous ´TT´. We purified the PCR product and then saw the result after single digestion by *TaqI* restriction enzyme .No difference found in the result.

Conclusion

The present study showed that the VDR polymorphism in *Bsm*I region is associated with T1DM in the Saudi population. As minor result; we find that ´tt´ genotype is rare in our Saudi volunteers. We recommend using the same methodology with larger sample size in the same population. In addition, future studies on the correlation between environmental factor such as UV, immune response, VDR SNPs and T1DM may also be considered.

Ethical APPROVEAL

The study was approved by Scientific Committee and the ethics Committee of Scientific Research that are registered at the National Commission for bio-medical ethics with number (HA-02-J-008),Jeddah –Saudi Arabia:date of approval 24 May 2015 ,referance number :169-14.

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