International Journal of Pharmacology

Research Article
Relationship Between Vitamin D Receptor Gene Polymorphisms and Type 1 Diabetes Mellitus in Saudi Patients

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Abstract

Background and Objective: Type 1 diabetes mellitus (T1DM) is considered as an autoimmune disease, in which both environmental and genetic factors are involved. Vitamin D (Vit D) is a vital hormone that plays an important role in immune system homeostasis, acting through vitamin D receptor (VDR). Gene polymorphisms in VDR were shown to be associated with immune diseases. The aim of this study was to look for a possible association between T1DM and the polymorphisms in VDR gene at the sites namely *TaqI*, *BsmI* and *ApaI*. **Materials and Methods:** A total of 50 T1DM patients (F = 25, M = 25) were studied. Patients and 50 age matched controls were genotyped for *BsmI*, *ApaI* and *TaqI* polymorphisms using PCR-RFLP assay. Statistical analyses were performed using MegStat® version 9.0 computer program. Differences in distribution of the genotypes were compared using one-way Analysis of Variance (ANOVA). **Results:** The results of the present study clearly showed for the first time, that there is an association between VDR gene polymorphisms and T1DM at the *BsmI* site in Saudi diabetic patients. **Conclusion:** This study could help us to establish the role of VDR gene in occurrence of T1DM. This would help us to develop a pharmacogenomic approach towards treatment of diabetes in relation to serum vit D levels.

INTRODUCTION

Type 1 Diabetes Mellitus (T1DM) is an autoimmune disease in which the immune system attacks and destroys the beta cells. People with this type of diabetes require insulin injections for survival. There are many complications related to Diabetes Mellitus (DM) such as dehydration, weakness and fatigue, vaginal or penile yeast infection, weight loss, blurred vision and confusion[1]. The long-term complications are categorized into 2 types: microvascular and macrovascular. The microvascular relate to the small blood vessels and capillaries and lead to kidney, eye and nerve disease. The macrovascular complications relate to disease of medium-sized and large blood vessels and lead to heart attacks, circulation problems in the legs and strokes[2].

Vitamin D (Vit D) plays a major role in the development and maintenance of bone tissue and also in maintaining normal homeostasis of calcium and phosphorus. Recently, vit D has been associated with metabolic syndrome[3]. The 1,25-dihydroxyvitamin $D_3[1,25(OH)_2D_3]$ plays a major role in affecting components of the immune system[4]. Vitamin D is obtained both through dietary intake (10-20%) as well as cutaneous synthesis on exposure to sunlight (80-90%)[5]. Vitamin D_3 or cholecalciferol and vitamin D_2 or D_3 undergo hydroxylation in the liver, leading to formation of 25-hydroxyvitamin D or 25(OH)D, that is the chief circulating form. About 25(OH)D then undergoes hydroxylation in the kidney with the help of 1α-hydroxylase enzyme to form the biologically active, dihydroxylated form of vit D, calcitriol or $1,25(OH)_2D$, which acts through specific vit D receptors to regulate not only calcium metabolism, but also is involved in the differentiation of various cell types[6].

It is postulated that in addition to its major role in bone mineralization, calcium homeostasis and related disorders, vit D may play a role in muscle strength[7-9], pathogenesis of psoriasis[10], certain cancers[11,12], Multiple Sclerosis (MS)[13], diabetes[14] and blood pressure, among other physiological and pathophysiological processes, all these indicating the high importance of maintaining normal levels of serum vit D to lead a healthy life. It is very challenging to determine vit D status of a population.

Vitamin D exerts its genomic action via the nuclear vitamin D receptor (VDR), that exhibits significant polymorphism. The highly polymorphic VDR gene is located on chromosome 12q (12-12q14). The VDR is expressed widely in many cell types, such as lymphocytes, macrophages and pancreatic-cells[15]. Four major polymorphic sites have been described within the VDR gene. Polymorphic *BsmI* and *ApaI* sites are present in intron 8 and a silent T to C substitution creates a *TaqI* restriction site in exon 9[16]. Investigation on association between some VDR gene polymorphisms and DM have shown that they influence susceptibility to DM in many populations[17,18]. It has been well established that a length of the VDR, affected by the presence of the polymorphisms, could result in lower activation of target cells, as a longer VDR protein seems to have a decreased transcriptional activity[19,20].

The aim of the present study was to look for a possible correlation between polymorphisms in the VDR gene and the occurrence of T1DM in Saudi patients. The results of the study will help to understand the molecular mechanism of interaction between vit D status and metabolic disorders such as DM. This information will help us in designing a pharmacogenomic approach towards treatments of DM making it more effective.

MATERIALS AND METHODS

Sample collection: Blood samples were collected from King Abdulaziz University Hospital (KAUH) from 50 control subjects with normal blood glucose levels and 50 type 1 diabetic patients. Informed consent was taken from all study subjects to participate in the research. The study was carried out between 2015 and 2016 a King Fahd Medical Research Center (KFMRC). Fasting glucose was measured by Dimension Vista® System (Siemens, Germany) and vitamin D (Total) levels was measured using ADVIA Centaur® immunoassay System (Siemens, USA). A K_2-EDTA tube was used for DNA extraction from whole blood. All chemicals used in the study were of analytical grade.

DNA extraction: Genomic DNA was extracted from whole blood samples using QIAamp DNA Blood Mini Kit (QIAGEN, USA, Cat. No. 51104). The extracted DNA was stored at -20°C for PCR amplification. The concentration and purity of the extracted DNA was calculated automatically by Nanodrop2000c instrument from Thermo Scientific (USA).

Polymerase chain reaction: For Polymerase Chain Reaction (PCR), the master mix from Thermo Scientific (Maxima Hot Start Green PCR Master Mix (2X), K1061, USA) was used. The primers were purchased from Biolegio, Netherlands. The forward primer was (5'-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3') and the reverse primer was (5'-GCA ACT CCT CAT GGC TGA GGT CTC-3') according to Uitterlinden *et al.*[21]. The 100 µM stock of these primers was prepared according to the manufactures instructions and then 10 µM aliquots were prepared by 1:10 dilution using sterile distilled water.

Table 1: PCR reaction components

Components	Final concentration	Quantity (μL)
Maxima Hot Start Green PCR Master Mix (2X)	2X	25
Forward primer (10 μM)	0.2 μM	4
Reverse primer (10 μM)	0.2 μM	4
Template DNA	1 μg	12
Water, nuclease-free (R0581)		5
Total		50

For PCR, the reaction mixer (50 μL) contained 2X reaction buffer (containing 500 mM KCl, 100 mM tris-HCl pH 8.3 and 1% Triton X-100 final concentrations), 4 mM Mg^{2+} 4 μM deoxyribonucleoside triphosphates, 0.2 μM of each primer, 0.45 U Taq DNA polymerase and 10-30 ng of DNA template. The total reaction volume was made up to 50 μL with nuclease free water (Table 1).

The master mix and the sample were divided into the PCR tubes in pre-PCR area before transferring them to the thermal cycler GeneAmp® PCR System 9700 (Model No. N805S3052708) supplied from Applied Biosystem, Japan. The amplification conditions consisted of an initial denaturation at 95°C for 4 min, 30 cycles of denaturation at 95°C for 30 sec, an annealing at 60°C for 1 min and an extension at 68°C for 2 min, followed by a final extension at 72°C for 5 min and ended at hold at 4°C.

To verify PCR product, horizontal gel equipment (Model No. 48205) and an electrophoresis power supply (Model No. 041BR) from Bio-Rad, UK were used. The amplification products were resolved in 1% agarose ethidium bromide stained gels and 1X Tris-borate-EDTA (TBE) electrophoresis buffer. The gel of electrophoresis was prepared using agarose, ethidium bromide promega (USA) and TBE buffer (10X) which were purchased from Thermo Scientific, US. DNA loading dye (R0611) and DNA ladder (SM0314) ranging from 250-10,000 bp were used in each run. Gel documentation system (Model No.M03 2746) from UVitec, Cambridge, UK, was used to visualize the PCR product.

RFLP analysis of VDR gene polymorphism: Amplified PCR products (5 μL) were digested with 3000 U of each restriction enzyme from Thermo Scientific, USA. These enzymes are *ApaI*, *BsmI* and *TaqI.* These enzymes were used according to the supplier-recommended protocols (Thermo Scientific, US, Canada). By using the thermal cycler, the reaction was incubated in 37°C for 1 h 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 h which is the activation temperature of *TaqI*, then the enzymes were inactivated by incubation at 80°C for 20 min. The RFLP products were electrophoresed on 2% agarose gel. All chemicals used were of analytical grade and were used as received without any further.

Statistical analysis: All statistical analyses were performed using the MegStat® version 9.0 computer program. Data were given as Mean±Standard Deviation (SD). Differences among the two groups were tested using the t-test. Differences in distribution of the genotypes were compared using one-way Analysis of Variance (ANOVA). Rare genotypes (n = 0 in any group) were excluded from the analysis. Differences were considered statistically significant for p-value <0.05[22].

RESULTS

The volunteers in this study were classified according to Fasting Blood Glucose (FBG) test as two groups: T1DM and the control group. Each group consisting of 25 females and 25 males, total 50 subjects each matched in age. The clinical and biochemical parameters for the two groups, T1DM and control sample are shown in Table 2. The age between T1DM and the control group showed significant differences (p<0.05), but non-significant differences between females and males in the 2 groups. The FBG values between T1DM and control subjects shows significantly different at p<0.05 but non-significant difference between females and males in the 2 groups. The vit D total values between each of the 2 groups, females and males, were not significantly different (p>0.05) between T1DM and control subjects.

The PCR products in our samples from Saudi volunteers, ~2229 bp as shown in Fig. 1, were digested with the *BsmI, TaqI* and *ApaI* restriction enzymes. Accordingly, the allele frequencies were 28% for B and 72% for b in *BsmI,* 50% for A and 50% for a in *ApaI* and 52% for T and 48% for t in *TaqI* RFLPs. The most frequent genotype was the recessive homozygous genotype in *BsmI* (60%). Interestingly, in *BsmI* genotype, the homozygous recessive was more frequent in diabetic people compared to control subjects, with bb frequency of 60% in diabetics compared to 24% in the control group (Table 3).
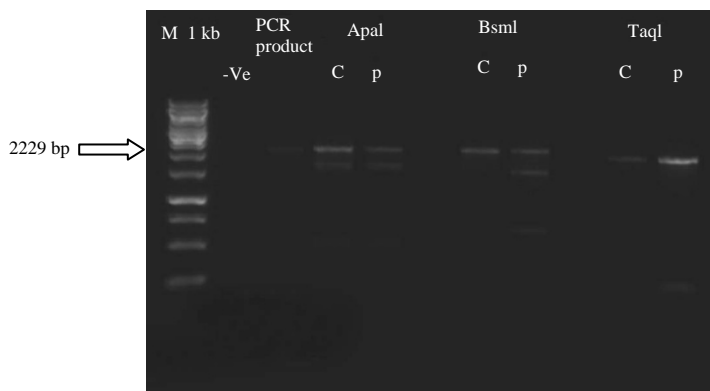
Fig. 1: One percent agarose gel showing the results of three restriction enzymes *Apal, Bsml* and *Taql*

Lane M: DNA marker, Lane 2, 3: Negative control and PCR products yielded one band of size 2229 bp, Lane 4-8: Comparisons between three restriction enzymes *Apal, Bsml, Taql* according to T1DM (P) and the control group (C)

Table 2: Clinical and biochemical characteristics of type 1 diabetes mellitus (T1DM) and controls

Parameters	Control (Mean±SD)		T1DM (Mean±SD)		p^1	p^2	p^3
	Female (n = 25)	Male (n = 25)	Female (n = 25)	Male (n = 25)			
Age (years)	26.27±3.810	24.44±4.20	23.17±5.190	23.17±5.19	>0.05	>0.05	>0.05
	25.37±4.070		23.44±5.380				
Vitamin D (total) (nmol L^{-1})	40.88±21.39	33.04±14.60	39.00±24.89	40.65±18.31	>0.05	>0.05	>0.05
	37.04±18.62		39.66±22.30				
FBG mmol L^{-1}	5.34±0.500	5.48±0.39	12.01±6.220	12.05±5.78	>0.05	>0.05	<0.05
	5.41±0.450		12.02±6.020				

FBG: Fasting blood glucose, Values represented as the Mean±Standard Deviation (SD), p^1: Value for female and male control groups, p^2: Value for female and male T1DM, p^3: Value for control groups and T1DM

Table 3: Distribution of the genotypes in the control and diabetic patients

Restriction sites	Genotypes	Control (n = 50)	Frequency (%)	T1DM (n = 50)	Frequency (%)
Apal	AA	18	36	17	34
	Aa	16	32	16	32
	aa	16	32	17	34
Bsml	BB	26	52	8	16
	Bb	12	24	12	24
	bb	12	24	30	60
Taql	TT	16	32	19	38
	Tt	16	32	14	28
	tt	18	36	17	34

DISCUSSION

This study has revealed for the first time, that there is an association between VDR gene polymorphisms and T1DM at the *Bsml* site in Saudi diabetic patients. The majority of T1DM is considered as a T-cell-mediated autoimmune disease and vit D compounds are well known to supress T-cell activation via the binding to VDR and thus VDR gene polymorphism were shown to associated with immune diseases[23]. Therefore, the aim of present study was to investigate the possible relationship between polymorphisms in the VDR gene and occurrence of T1DM in Saudi population. As far as the authors are aware, this was the first study that showed a positive relationship between T1DM and VDR gene polymorphisms in Saudi patients. Results from the present study demonstrated that B allele of *Bsml* gene polymorphisms was more frequent in healthy controls compared with T1DM patients and there was a highly significant difference (p<0.05) in allele frequency between the cases and the controls. In control subjects, the frequency of homozygous recessive genotype bb was only 24% while in T1DM patients it was 60%. Therefore, allele b in *Bsml* region is a risk allele. A similar study carried out on Japanese population by Motohashi *et al.*[23] analyzed the polymorphism at the *Bsml* site on the VDR gene and found that *Bsml* polymorphism is associated with acute-onset type 1 diabetes. In agreement with our results, a study on Taiwanese, has shown that T1DM was associated with B allele[24].

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 patients. In contradictory to our results, a recent study on Egyptian population has shown that *ApaI* and *TaqI* VDR gene polymorphisms have an association with T1DM[25]. In Saudi population, a study showed that *ApaI* was found to be in low frequency in T1DM[26].

This outcome matches the outcome of the largest metadata which found relationship between *BsmI* polymorphisms in VDR and T1DM, whereas the *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[27]. 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[28]. A recent study by Iyer *et al.*[29], found that *ApaI* polymorphism of VDR gene is associated with type 2 diabetes mellitus in Saudi population.

Furthermore, the variations of the results between this study and other studies might be due to ethnic differences and the interactions with other genetic or environmental factors that are involved in the pathogenesis of T1DM[30]. Recently, it was observed that many people do not respond to standard diabetic drugs[31]. Though many pharmacogenomics researches have described a relationship between single nucleotide variations and drug resistance, there are still conflicting results[32]. Thus, performing a pharmacogenomics analysis that might help us in the long run to formulate and administer the correct anti-diabetic drug according to the patient's genetic variation of the VDR gene.

CONCLUSION

The results of the present study clearly demonstrated that the B allele of *BsmI* polymorphism in the VDR gene is associated with increased risk of T1DM in Saudi patients. The homozygous recessive genotype bb seems to be more predominant in diabetic patients while the homozygous dominant genotype BB is less frequent in diabetic patients indicating that the B allele clearly offers protection against T1DM. This study is an important milestone in establishing the correlation between a particular SNP and prognosis of T1DM and will pave the way to develop a pharmacogenomic approach towards appropriate treatment of T1DM.

SIGNIFICANCE STATEMENTS

This study has clearly shown that the *BsmI* allele of the vitamin D receptor gene is significantly associated with T1DM in Saudi population. Although many studies have been carried out previously on the vitamin D receptor gene polymorphism's possible role in T1DM, results have been very different in various ethnic groups. This study has revealed that the homozygous recessive genotype bb is associated with T1DM and this is an important finding that throws light on the protective role of the dominant B allele of *BsmI* site in healthy controls, making it a candidate gene for further pharmacogenomic studies in the prognosis and treatment of T1DM.

ACKNOWLEDGMENTS

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