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ASSOCIATION OF *Apa I* POLYMORPHISM OF VITAMIN D RECEPTOR GENE WITH TYPE 2 DIABETES MELLITUS IN SAUDI POPULATION

Archana P. Iyer^{1,2,*}, Susan Lanham New^{2,3}, Sawsan Khoja^{1,2}, Maryam A.AL-Ghamdi^{1,2} and Sami Bahlas^{2,4}

¹Department of Biochemistry, King Abdulaziz University, Jeddah, Saudi Arabia

²Vitamin D Pharmacogenomics Research Group, King Abdulaziz University, Jeddah, Saudi Arabia

³Health and Medical Sciences College, Department of Nutrition and Metabolism, University of Surrey, United Kingdom

⁴Department of Internal Medicine, College of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia

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ABSTRACT

Vitamin D deficiency has been associated with the acceleration of the onset of T2DM. The biological effects of vitamin D are mediated by binding to the vitamin D receptor (VDR) which belongs to the steroid receptor superfamily. Although many polymorphisms exist in the VDR gene, their effect on VDR protein function and signalling is unknown. An association between VDR polymorphism and T2DM has been reported in some studies; however, it appears to vary across different populations around the world. Hence this study was carried out to investigate the relationship between VDR gene polymorphisms at three restriction sites *ApaI*, *BsmI*, and *TaqI* and the risk of T2DM in Saudi population. The volunteers were classified according to Fasting Blood Glucose (FBG) test as two groups, T2DM and normal group. The VDR gene was amplified by polymerase chain reaction (PCR). PCR products were digested using restriction enzymes: *ApaI*, *TaqI*, and *BsmI* and the bands were visualised by agarose gel electrophoresis and ethidium bromide dye under UV. The results of the current study showed a significant difference in genotypes and allele frequencies of the VDR gene polymorphisms at the *ApaI* site between T2DM patients and control groups.

* Corresponding author

E-mail: arch729@gmail.com (Archana P. Iyer)

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1 Introduction

Type 2 diabetes mellitus (T2DM) is a public health problem. It is estimated that there are over 347 million people living with diabetes worldwide, most of them having T2DM (Whiting et al., 2011). T2DM is a leading cause of premature death, mainly from cardiovascular diseases (Van Dieren et al., 2010). Globally, it has been estimated that 285 million people worldwide have diabetes, the majority of them have T2DM (International Diabetes Federation, 2011).

The mechanisms involved in prognosis of T2DM have been considered to be influenced by genetic factors (Zhang et al., 2016). Numerous studies have been carried out worldwide to study the genes that attribute to susceptibility to (Malecki et al., 2003; Herder et al., 2008; Wu et al., 2008; Bid et al., 2009; Dilmec et al., 2010; Anouti, 2013). Deficiency of vitamin D is a worldwide public health issue. A study by James in 2008, reports that over 1 billion individuals suffer either from insufficient or deficient levels of vitamin D. Further, the association between vitamins D deficiency and abnormal glucose metabolism as seen in T2DM has been shown by various researchers (Melamed et al., 2008; Palomer et al., 2008; Pittas et al., 2010; Al-Shoumer & Al-Essa, 2015). A direct action via vitamin D receptor (VDR) gene activation or indirect action via calcemic hormones or inflammation was demonstrated by Thorand et al. (2011) and Sung et al. (2012).

The active form of vitamin D (1,25(OH)₂D₃) has important immunomodulatory properties and influences insulin secretion (Norman et al., 1980). Vitamin D₃ acts through its specific receptor, a nuclear protein called VDR. The VDR gene is located on chromosome 12q (12–12q14) (Nejentsev et al., 2004). 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 (Uitterlinden et al., 2004; Panierakis et al., 2009). Activation of T-cell is suppressed by VDR, thus polymorphisms in this receptor gene are associated with T-cell mediated autoimmune diseases (Motohashi et al., 2003). Various polymorphisms have been studied in detail with respect to the VDR genes.

Polymorphisms such as *BsmI* and *FokI* have been demonstrated in altering the activity of VDR protein (Filus et al., 2008). Some polymorphisms in the VDR gene were identified by allelic variation in restriction enzyme sites and are located between exons 8 and 9 such as *Tru9I*, *TaqI*, *EcoRV*, and *ApaI* (Naito et al., 2007). *FokI* polymorphism which is located in exon 2 has been shown to have functional role in transcriptional activation of VDR gene (Whitfield et al., 2001). However, the genetic background of the disease still remains unclear. In present work, author studied the *ApaI*,

BsmI and *TaqI* restriction enzyme polymorphisms of the VDR gene in patients with T2DM in Jeddah, Saudi Arabia.

2 Materials and Methods

All subjects routinely attended diabetic clinic, Association of Diabetic Patient Friends Jeddah, King Abdulaziz University Hospital (KAUH), Jeddah, Saudi Arabia. T2DM patients were diagnosed according to the guide line proposed by World Health Organization (WHO, 2006). All the Studied subjects were classified into two groups, 50 control subjects and 50 T2DM patients. The control group consisted of 25 males and 25 females with the age ranged from 37 to 60 years and they had overnight fasting blood glucose levels of <110 mg/dl. Further, T2DM patients group were also have 25 men and 25 women with the same age group and fasting blood glucose. The study was approved by the unit of biomedical ethics research committee (UBERC - Reference No. 169-14), King Abdulaziz University Hospital (KAUH), Jeddah, and informed consent was obtained from all study participants.

2.1 Blood biochemistry analyses

Blood samples were drawn from the subjects after at least 8 h of fasting. Two sets of fasting blood samples were collected separately from each subject in clot activator and gel tubes, for glucose and total vitamin D. For DNA extraction, a spray coated K₂ EDTA tubes were used. Fasting glucose was measured photometrically according to the manufacture's instructions using the Dimension Vista ® System (Siemens AG, Erlangen, Germany). Vitamin D Total (VitD) was measured by ADVIA Centaur ®immunoassay System (Siemens, USA) (Bid et al., 2009).

2.2 Extraction of DNA and amplification of vitamin D receptor gene

Whole blood was used to extract genomic DNA using QIAamp DNA blood mini kit (QIAGEN, USA, Cat.no.51104). The extracted DNA was stored at -20°C for further PCR reactions. On the other hand, concentration and purity of the extracted DNA was calculated automatically by Nanodrop2000c instrument from Thermo Scientific (USA). For Polymerase Chain Reaction (PCR), the reactions were prepared using Maxima Hot Start Green PCR Master Mix (2x). The primers were purchased from Biolegio, Nijmegen, 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'). For polymerase Chain Reaction (PCR), the master mix from Thermo Scientific (Maxima Hot Start Green PCR Master Mix (2X), K1061, USA) was used. The reaction mix (50 µl) contained 2X reaction buffer, 4 mM Mg²⁺, 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.

Table1 Serum vitamin D levels

Group	Serum vitamin D levels ng/ml		
	<20 (deficient)	20-30 (insufficient)	>30 (sufficient)
Control group (n=50)	5	35	10
T2DM (n=50)	41	9	0

The amplification conditions consisted of an initial denaturation at 95°C for 4 min, 30 cycles of denaturation at 95°C for 30 S, 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 (Uitterlinden et al., 1996).

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 of Tris- borate- EDTA (TBE) buffer.

2.3 Genotyping of the VDR gene

The amplified PCR product of ~2229 bp was digested with three restriction enzymes namely *ApaI*, *BsmI*, and *TaqI*, obtained from Thermo Scientific. In each reaction, 5 µl equivalent to about 0.5 µg of purified PCR product was digested with 10 units of each restriction enzyme according to the optimal temperature and conditions of each respective enzyme. For *ApaI* and *BsmI*, it was 37°C for 1 h and inactivation at 65° C for 20 min, for *TaqI* it was 65° C for 1 h and inactivation at 80° C for 20 min (Malecki et al., 2003). The restriction enzyme digested products were run on 1% agarose bromide stained gels and 1X TBE electrophoresis buffer.

3 Results

3.1 Serum vitamin D levels in association with diabetes

While comparing the vitamin D levels between control subjects and T2DM patients, results of the present study revealed that the serum vitamin D levels were significantly lower in T2DM patients as compared to the controls as indicated in table1, wherein, only 10% of the control group was deficient, while 82% of the T2DM group was deficient.

3.2 PCR-RFLP analyses and distribution of the genotypes

Based on the PCR-RFLP, different patterns were reported for the three restriction sites. The sizes of the RFLP products were as follows: for *ApaI*, the sizes were 1700 and 259 bp for aa, 2229 bp for AA, and 2229, 1700, and 529 bp for Aa. Regarding *BsmI*, the sizes were 1579 and 650 bp for bb, 2229 for BB, and 2229, 1579, and 650 bp for Bb. In *TaqI*, the sizes for tt were 1780, 202, and 247 bp, for TT the sizes were 1982 and 247, and for Tt the sizes were 1982, 1780, 202, and 247 bp. The PCR-RFLP results are indicated in figure 1.

Figure 1 Photograph of a 1% agarose gel showing the results of three restriction enzymes *ApaI*, *BsmI* and *TaqI* digestion. Comparison between the three restriction enzymes in T2DM (P) and the control (C). Lane M: DNA marker. Lane 2 and 3: negative controls and PCR products yielded one band of size 2229 bp. Lane 4, 5, 6, 7 and 8.

Table 2 Distribution of various alleles in control subjects

Restriction site	Genotype	Number of samples	% frequency
<i>ApaI</i>	AA	17	34
	Aa	21	42
	Aa	12	24
		(50 total)	(100% total)
<i>BsmI</i>	BB	21	42
	Bb	16	32
	Bb	13	26
		(50 total)	(100% total)
<i>TaqI</i>	TT	11	22
	Tt	21	42
	Tt	18	36
		(50 total)	(100% total)

Table 3 Distribution of various alleles in T2DM patients

Restriction site	Genotype	Number of samples	% frequency
<i>ApaI</i>	AA	2	4
	Aa	7	14
	Aa	41	82
		(50 total)	(100% total)
<i>BsmI</i>	BB	10	20
	Bb	25	50
	Bb	15	30
		(50 total)	(100% total)
<i>TaqI</i>	TT	14	28
	Tt	19	38
	Tt	17	34
		(50 total)	(100% total)

Based on the PCR-RFLP results, studied respondents can be divided into the control subjects as well as the T2DM patients according to their genotypes at the different SNPs as depicted in tables 2 and 3. Moreover, these results revealed that the frequency of occurrence of the homozygous recessive genotype aa was higher in diabetic patients compared to the control group.

4 Discussions

Vitamin D deficiency as well as type 2 DM has been genetically influenced to a great deal. Many genetic variants have been identified as related to T2DM as shown by GWAS (genome wide association studies). These variants are highly correlated to population-based determinants which contribute to high risk in specific ethnic groups, leading to a significant increase in prevalence of the disease (Anouti, 2013). This research of ours reports a significant difference in allele frequency and genotype pattern of the VDR gene at the *ApaI* site comparing T2DM cases and control population. To best of our knowledge, this is the first report of association between *ApaI* polymorphisms and T2DM in Saudi population. Various studies from around the world have shown associations between VDR gene polymorphisms and risk of developing T2DM.

However, the results are inconsistent and inconclusive and variable according to the populations and ethnic groups studied. A study conducted in Asian population by Li et al. (2013), suggested that there might be a correlation between polymorphisms at the *FokI* site and onset of T2DM. A study in North Indian population conducted by Bid et al. (2009), was aimed at analyzing association between polymorphisms of VDR gene and genetic predisposition to T2DM. They evaluated SNPs of *FokI* (T/C) [rs2228570], *BsmI* (A/G) [rs1544410] and *TaqI* (C/T) [rs731236] using PCR-RFLP technique similar to this study, but did not find any significant association between the polymorphisms at any of the sites studied between patients and controls. Malecki et al. (2003) similarly studied the association of *FokI*, *ApaI*, *BsmI*, and *TaqI* polymorphisms of VDR gene with T2DM in a Polish population, but they too did not find any correlation between VDR gene polymorphisms and diabetes at any of the four polymorphic sites. Similarly, studies conducted by Dilmec et al. (2010), did not provide any evidence for the association of VDR polymorphisms at *ApaI* and *FokI* sites with T2DM in a Turkish population.

A recent metadata analysis conducted by Yu et al. (2016), concluded that there is no significant association between *BsmI* and *FokI* polymorphisms and T2DM, but the results vary according to sample size and ethnicity of the study population. Considering the

above studies, present study is very encouraging in the fact that it has shown that the *Apal* site has a strong correlation between VDR gene and T2DM, which has not been observed in many other studies from different populations. Allele distributions, ethnic background, exchange of gene pool are major reasons affecting this type of a result. It is well known that there is a large genetic exchange in Saudi population with high amount of cross breeding with neighboring countries such as Egypt, Yemen, Syria etc which lead to a wide spectrum of genetic allele frequency combinations which might be a major contribution towards the observed result. Extending this study to a larger sample size would confirm this theory

Conclusion

The results of the current study showed a significant difference in genotypes and allele frequencies of the VDR gene polymorphisms at the *Apal* site between T2DM patients and control groups. Formulating population-based genetic association studies between VDR polymorphisms and T2DM will be a major step towards elucidating the underlying mechanism at the genetic level that leads to onset of T2DM in a large percentage of the population as well as vitamin D deficiency.

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Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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