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ASSOCIATION OF VITAMIN D RECEPTOR GENE POLYMORPHISMS AND GESTATIONAL DIABETES IN SAUDI WOMEN

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ABSTRACT

Vitamin D is primarily associated with a major role in bone formation. It is also associated with onset and prognosis of diseases such as diabetes, cancer and cardiovascular diseases. In Saudi Arabia, the prevalence of DM is one of the highest reported in the world. There is accumulating evidence suggesting an association between the DM and vit D deficiency. This study was proposed to establish a possible correlation between gestational diabetes mellitus (GDM) and the polymorphisms in the vitamin D receptor (VDR) gene at the sites namely *TaqI*, *BsmI*, and *ApaI*. Results of this study revealed that homozygous recessive genotype tt of the *TaqI* site with GDM. This study will help to establish the role of VDR gene in occurrence of various subsets of DM which is a major metabolic disorder in Saudi Arabia.

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1 Introduction

Gestational diabetes is one of the most frequent conditions associated with diabetes causing severe maternal and neonatal complications (Blumer et al., 2013). The frequency of Gestational diabetes mellitus (GDM) usually reflects the frequency of type 2 diabetes in the underlying population. Women who have a history of GDM are at a higher risk for subsequent type 2 diabetes (Moses, 2012). There are many complications related to DM such as dehydration, weakness and fatigue, vaginal or penile yeast infection, weight loss, blurred vision and confusion. Long-term complications are categorized into two types viz microvascular and macrovascular. The microvascular relate to the small blood vessels and capillaries and lead to kidney, eye, and nerve disease (Hapo study, 2008). While, macrovascular complications relate to disease of medium-sized and large blood vessels and lead to heart attacks, circulation problems in the legs, and strokes (Masharani, 2008). GDM is a major health concern affecting a great share of pregnancies, with complications arising from abnormally high blood glucose levels (American Diabetes Association, 2011). According to the recommendation of the World Health Organization (WHO), increased blood glucose levels in pregnancy are termed as gestational diabetes or diabetes mellitus (WHO, 2013). During maternal diabetes, fetal insulin levels are increased that promote the storage of excess energy as fat and act as a growth factor and causing macrosomia (Dabelea et al., 2000). Mothers with GDM have up to 50% chance of developing T2DM within 5 years of delivery.

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, various reports have shown the relationship between metabolic syndrome and deficiency of vitamin D (Wimalawansa, 2016). Further, 1,25-Dihydroxyvitamin D₃[1,25(OH)₂D₃] also plays a major role in affecting components of the immune system (Holick, 2002). Vit D is obtained both through dietary intake (10–20%) as well as cutaneous synthesis on exposure to sunlight (80–90%) (Rizzoli & Bonjour, 2004). Vit D₃ or cholecalciferol, and vitamin D₂ or D₃ is hydroxylated in the liver, leading to formation of 25-hydroxyvitamin D or 25(OH)D, which is the chief form in circulation. 25(OH)D is then hydroxylated in kidney with the aid of 1 α -hydroxylase forming the biologically active, dihydroxylated form of vit D, calcitriol or 1,25(OH)₂D, which acts through specific vit D receptors to regulate calcium metabolism and the differentiation of various cell types.

The genomic action of vitamin D is expressed through the vitamin D receptor (VDR), that shows high degree of polymorphism. The VDR gene is located on chromosome 12q (12-12q14). The expression of VDR is noted in various cells, such as lymphocytes, macrophages, and pancreatic-cells (Pittas et al., 2007). Detailed description of four polymorphic sites have been widely studied. A polymorphic *FokI* site in exon 2

produces an alternative transcription initiation site, causing the formation of a different protein with three amino acids being added to the amino terminus. 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 (Rostand, 1979). Investigations on association between some VDR gene polymorphisms and DM have shown that they influence susceptibility to DM in many populations (Dawson-Hughes et al., 2005; Jones et al., 2007). 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 (Baroncelli et al., 2008; Mithal et al., 2009).

The aim of the present study was to look for a possible correlation between polymorphisms in the VDR gene and occurrence of GDM in Saudi women. The study will help in understanding the molecular mechanism of interaction between vit D status and gestational diabetes. This information will help us in designing a pharmacogenomic approach towards treatment of different types of diabetes making it more effective.

2. Materials and Methods

2.1 Sample collection

Blood samples were collected from 50 pregnant women with normal blood glucose levels, and 50 women with gestational diabetes. All the subjects were well informed and their written consent was taken to participate in the research program. Two sets of fasting blood samples were collected separately from each subject in clot activator and gel tubes (for Glucose and Vit D Total) and Spray-coated K₂EDTA plastic tubes (for DNA extraction). The serum was separated and stored at -80°C until further analysis. Fasting glucose was measured by Dimension Vista® System (Siemens, Germany). Total vitamin D was estimated using ADVIA Centaur® immunoassay System (Siemens, USA). DNA extraction was done from whole blood and stored at -20°C till further manipulations.

2.2 DNA extraction

Genomic DNA was extracted from whole blood samples, in biosafety cabinet, using QIAamp DNA Blood Mini Kit (QIAGEN, USA, Cat.no.51104). Storage of DNA was at -20°C for PCR amplification. On the other hand, concentration and purity of the extracted DNA was calculated automatically by Nanodrop2000c instrument from Thermo Scientific (USA).

2.3 Polymerase chain reaction

For Polymerase Chain Reaction (PCR), the reactions were prepared using Maxima Hot Start Green PCR Master Mix (2X).

Table 1. The PCR Reaction Components

Component	Final concentration	Quantity
Maxima Hot Start Green PCR Master Mix (2X)	X2	1µ 25
Forward Primer (10 µM)	Mµ 0.2	1µ 4
Reverse Primer (10 µM)	Mµ 0.2	1µ 4
Template DNA	1 µg	1µ 12
Water, nuclease-free (R0581)		1µ 5
Total		1µ 50

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'), as used by Uitterlinden et al. (1996). The 100 µM stock of these primers was prepared according to the instruction of their company and then 10 µM aliquots were prepared by 1:10 dilution with distilled water.

For PCR, Thermo Scientific (Maxima Hot Start Green PCR Master Mix (2X), K1061, USA) was used. The reaction mix (50 µl) consisted of 2X reaction buffer, 4 mM Mg²⁺, 4 µM deoxyribonucleoside triphosphates, 0.2 µM of each primer, Taq DNA polymerase to a concentration of 0.45 U and template DNA concentration of 20 ng, and sterile water making up the final volume (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 PCR conditions consisted of initial denaturation at 95°C for 4 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing temperature of 60°C for 1 min, then an extension at 68°C for 2 min, followed by a final extension at 72°C for 5 min and final hold at 4°C.

PCR product was verified by horizontal gel equipment (Model No.48205), and an electrophoresis power supply (Model No.041BR) from Bio-Rad, UK. The visualization of PCR products was carried out by electrophoresis on 1% agarose gels using 1X TBE 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 to 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.

2.4 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 used for digestion were *Apal*, *BsmI*, and *TaqI*. These enzymes were used according to the supplier-recommended protocols (Thermo Scientific, US, Canada). In the thermal

cycler, the reaction was incubated for 1 hour at 37° C being the activation temperature of *BsmI* and *Apal*. Enzyme inactivation was achieved by incubation at 65°C for 20 min. This was followed by incubation at 65°C for 1 h to inactivate *TaqI*, followed by a final step of inactivation at 80°C for 20 min. RFLP products were separated on 2% agarose gel.

2.5 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. Mean of data was compared for different alleles and genotypes by analysis of variance (ANOVA). Rare genotypes (n=0 in any group) were excluded from the analysis. Differences in distribution of the genotypes between the females and males according to T1DM, T2DM, GDM in comparison to control subjects were examined with the chi-square analysis. Differences in genotype frequencies were considered statistically significant for *p*-value < 0.05.

3 Results

The volunteers in this study were classified according to Fasting Blood Glucose (FBG) test as two groups viz normal pregnant and GDM. The study group consisted of 50 pregnant women with normal blood glucose levels and 50 women with GDM. Result of study revealed that vit D levels in normal pregnant women was 38.32±18.37 nmol/ml, while in pregnant women with GDM, the levels were as low as 10.89±8.95 nmol/ml with a significant *p* value of < 0.01. The percentage vit D deficiency in normal women was 30 % while in women with GDM, it was as high as 87% with a significant *p* value of < 0.01.

The PCR products of the samples collected from Saudi volunteers, ~ 2229 bp as shown in Figure 1, were digested with the *BsmI*, *TaqI*, and *Apal* restriction enzymes. The allele frequencies for all three sites in control and GDM patients are represented in table 3 and 4 respectively. During study no significant difference was reported in the distribution of alleles at *Apal* and *BsmI* site between the two groups. Further, it was observed that the homozygous recessive genotype *tt* was higher accounting for 66% in GDM patients when compared to the control subjects.

Table 2. Clinical and biochemical characteristics of gestational diabetic women and controls.

Parameters	Normal pregnant women	Gestational diabetic women	P-value
	Mean $\pm$ SD (n=50)	Mean $\pm$ SD (n=50)	
Age (years)	29 $\pm$ 5.17	32 $\pm$ 6.29	0.104
Weight (Kg)	69.18 $\pm$ 14.94	81.09 $\pm$ 27.89	0.042
BMI	28.09 $\pm$ 5.31	32.21 $\pm$ 5.85	0.075
Gestational weeks	29.32 $\pm$ 6.2	29.57 $\pm$ 5.97	0.858
GCT (mmol/L)	6.4 $\pm$ 1.46	9.96 $\pm$ 2.03	0.000
GTTF (FBS) (mmol/L)	4.29 $\pm$ 0.66	5.32 $\pm$ 0.71	0.000
GTT1 (mmol/L)	7.61 $\pm$ 1.42	11.45 $\pm$ 1.28	0.000
GTT2 (mmol/L)	6.57 $\pm$ 1.22	10 $\pm$ 1.26	0.000
Vit D (nmol/L)	38.32 $\pm$ 18.37	10.89 $\pm$ 8.95	0.01
Positive History of diabetes %	54%	14%	—
Negative History of diabetes %	30%	6%	—
Unknown History of diabetes %	16%	80%	—

Values are mean $\pm$ SD. BMI, Body mass index; GCT, Glucose challenge test; GTTF, Glucose tolerance test fasting; FBS, Fasting blood sugar; GTT1, Glucose tolerance test after 1 hour; GTT2, Glucose tolerance test after 2 hour; *Highly significant difference ;Values represented as the mean $\pm$ standard deviation (SD); P Value for control and GDM group.

4. Discussion

GDM is a major health concern in pregnant women. Around the world, 1-20% prevalence of GDM has been reported, on par with obesity and type 2 diabetes mellitus (T2DM) (American diabetes association, 2011). DM is a group of metabolic disorders which is characterized by a chronic hyperglycemic condition resulting from defects in insulin secretion, insulin action or both. Although many studies have been carried out on the VDR gene and its association with various types of diabetes, the results seem to be highly population dependent (Iyer et al., 2017). Our study has clearly shown that the recessive t allele at TaqI site is associated with the prognosis of GDM. A similar study carried out on Saudi population by Tawfeek et al. (2011), analyzed the polymorphism at the BsmI site on the VDR gene and found no

correlation between vitamin D and GDM. Another study conducted on Iranian population by Aslani et al. (2011) on *FokI* polymorphism of the VDR gene clearly found a significant association between the F allele and prevalence of GDM. Further, they found that people who possessed the F allele had decreased incidence of gestational diabetes. In contrast to this result, the present study clearly indicates a role of the recessive t allele in development of GDM. Bid et al. (2009) performed a study on the same sites on an Indian population and did not report any association between the VDR gene polymorphism and type II diabetes. The fact that there are discrepancies between various studies could be attributed to the variation in gene pool among various ethnic groups in the VDR gene loci. Genetic and environmental interactions could also play a major role in the prognosis of diabetes.

Figure 1: Photograph of a 1% agarose gel showing the results of three restriction enzymes *Apal*, *BsmI*, *TaqI* and *FokI* digestion. 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 comparisons between three restriction enzymes *Apal*, *BsmI*, *TaqI* according to GDM (P) and the control subjects (C).

Table 3. Distribution of various alleles in control subjects

Restriction site	Genotype	Number of samples	% frequency
<i>ApaI</i>	AA	18	36
	Aa	20	40
	aa	12	24
		(50 total)	(100% total)
<i>BsmI</i>	BB	20	40
	Bb	14	28
	bb	16	32
		(50 total)	(100% total)
<i>TaqI</i>	TT	20	40
	Tt	15	30
	tt	15	30
		(50 total)	(100% total)

Table 4. Distribution of various alleles in GDM patients.

Restriction site	Genotype	Number of samples	% frequency
<i>ApaI</i>	AA	12	24
	Aa	16	32
	aa	22	44
		(50 total)	(100% total)
<i>BsmI</i>	BB	11	22
	Bb	17	34
	bb	22	44
		(50 total)	(100% total)
<i>TaqI</i>	TT	9	18
	Tt	8	16
	tt	33	66
		(50 total)	(100% total)

Conclusions

In this study, significant differences in vit D level were reported between pregnant women with normal blood glucose levels and diabetic women. Further, it was reported that people with lower than required levels of vitamin D serum levels are more prone to becoming diabetic. When considering the VDR gene polymorphisms, we found that the t allele of the *TaqI* polymorphic site was associated with increased risk of GDM. Both these findings are very important milestones in developing a pharmacogenomic approach towards treatment of diabetes. Unfortunately, the sample size of the study is small and hence we recommend that the study be extended to a larger population to arrive at a clearer picture.

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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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