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

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Received – xxxx; Revision – xxxx; Accepted – xxxx
Available Online – xxxx
DOI: http://dx.doi.org/10.18006/xxxxxx

KEYWORDS
Gestational diabetes mellitus
Gestational diabetes
Vitamin D receptor gene
Saudi Arabia
Polymorphism

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.
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 D, [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 1α-hydroxylase forming the biologically active, dihydroxylated form of vit D, calcitriol or 1,25(OH)2D3, 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 Fok1 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 Apal 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).
The primers were purchased from Biolegio, Netherlands. The forward primer was (5’-CAA CCA AGA CTA CAA GTA CCG CTG TCA CTG G-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 MgCl₂, 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. The gel of electrophoresis was prepared according to the documentation system (Model No.M03 2746) from Biolegio, Netherlands. The visualization of PCR products was carried out by electrophoresis on 1% agarose gel.

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

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 1. The PCR Reaction Components

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 the homozygous recessive genotype tt was higher in normal pregnant women compared to the 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 ± SD (n=50)      | Mean ± SD (n=50)            |         |
| Age (years)            | 29±5.17               | 32±6.29                     | 0.104   |
| Weight (Kg)            | 69.18±14.94           | 81.09±27.89                 | 0.042   |
| BMI                    | 28.09±5.31            | 32.21±5.85                  | 0.075   |
| Gestational weeks      | 29.32±6.2             | 29.57±5.97                  | 0.858   |
| GCT (mmol/L)           | 6.4±1.46              | 9.96±2.03                   | 0.000   |
| GTTF (FBS) (mmol/L)    | 4.29±0.66             | 5.32±0.71                   | 0.000   |
| GTT1 (mmol/L)          | 7.61±1.42             | 11.45±1.28                  | 0.000   |
| GTT2 (mmol/L)          | 6.57±1.22             | 10±1.26                     | 0.000   |
| Vit D (nmol/L)         | 38.32±18.37           | 10.89±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 ± 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

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).](http://www.jebas.org)
Table 3. Distribution of various alleles in control subjects

| Restriction site | Genotype | Number of samples | % frequency |
|------------------|----------|-------------------|-------------|
| ApaI             | AA       | 18                | 36          |
|                  | Aa       | 20                | 40          |
|                  | aa       | 12                | 24          |
|                  |          | (50 total)        | (100% total)|
| BsmI             | BB       | 20                | 40          |
|                  | Bb       | 14                | 28          |
|                  | bb       | 16                | 32          |
|                  |          | (50 total)        | (100% total)|
| TaqI             | 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 |
|------------------|----------|-------------------|-------------|
| ApaI             | AA       | 12                | 24          |
|                  | Aa       | 16                | 32          |
|                  | aa       | 22                | 44          |
|                  |          | (50 total)        | (100% total)|
| BsmI             | BB       | 11                | 22          |
|                  | Bb       | 17                | 34          |
|                  | bb       | 22                | 44          |
|                  |          | (50 total)        | (100% total)|
| TaqI             | 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.

Acknowledgement

This project was funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, under grant no. RG-8-130-36. The authors, therefore, acknowledge with thanks DSR for technical and financial support.

Conflict of interest

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

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