

## Associations of Epigenetic methylation with vitamin D receptor level in Iraqi Gestational diabetes mellitus patients

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### Abstract

**Background :**GDM (gestational diabetes mellitus) is a hazardous pregnancy situation that occurs when a pregnant woman who that have never had diabetes progresses to cause systemic hyperglycemia during their pregnancy.DNA methylation is an epigenetic mechanism it occurs in the vertebrate genomes and generates 5-methylcytosine by transferring a methyl group to the C5 position of cytosine. DNA methylation modulates gene expression by either attracting or preventing transcription factor(s) from binding to DNA. **Objective:** The purpose of this study is to figure out the connection between VDR-gene promoter methylation in Iraqi GDM and its consequence on vitamin D levels. **Materials and Methods:** This investigation comprised 50 patients with GDM (Group 1) and 30 healthy pregnant individuals as controls (Group 2). Methylation was analyzed by extracted DNA using Bisulfite conversion and detected by a specific primer. **Results:** The results of methylation effect on vitamin D3 expressed there were significant differences between methylated ( $25.74 \pm 2.00$ ) a percentage of 32(64.00%) of GDM samples compared with concentration ( $14.00 \pm 1.41$ ) of methylated sample control at the percentage of observed results of methylation (1 (3.3%) at p-value (0.00) while the Un-methylated and partial methylate GDM samples did not produce any significant effect comparing with healthy controls.

**Conclusions:** Due to the increased metabolic activity associated with pregnancy, it's imperative to keep glucose levels in balance. It is suspected that both genetic and epigenetic factors play a role in the development of GDM and that the underlying mechanisms are complex and alter over time. To create efficient treatments and preventative plans, a greater understanding of these mechanisms and how they affect GDM is needed.

**Keywords:** GDM, VDR gene, vitamin D., Epigenetic, methylation.

## 1. Introduction

A frequent pregnancy problem known as gestational diabetes mellitus (GDM) occurs when throughout gestation, hyperglycemia evolves at random. Always, hyperglycemia is caused therefore to inadequate glucose tolerance induced by malfunctioning of pancreatic cells against the backdrop of recurrent insulin resistance (1). Following the latest International Diabetes Federation (IDF) predictions for 2017, GDM affects around 14% of pregnancies globally (2). Deficiency of vitamin D throughout pregnancy has been connected to a variety of unfavorable neonatal outcomes along with an increased risk of troubles later in pregnancy. Epigenetics defines all mitotically and mitotically heritable changes in chromosome function/gene expression that are not coded in the DNA sequence itself. Recently, there has been a greater appreciation of the role of histone modification in vitamin D-mediated immune-regulatory and inflammation response (3). A recent study found a link between insufficiency of vitamin D and methylation modifications in leukocyte DNA; however, the abnormalities were negligible. According to the same study, patients with vitamin D deficiency were more likely to have decreased synthesis and higher catabolism of active vitamin D (4). The vitamin D system affects around 3% of the human genome and has pleiotropic activities (5). The careful regulation of the vitamin D system genes is critical for maintaining homeostasis. In organs other than that DNA methylation is an epigenetic method that takes place in the mammal genome, such as the introduction of a methyl group to the C terminal of cytosine to become 5-methylcytosine. Throughout development, the pattern of DNA methylation in the genome switches to a dynamic process among both de novo DNA methylation and demethylation. DNA methylation induces the expression by either binding proteins that affect genes or restricting transcription factor(s) from interacting with DNA. As a result, specialized cells express a constant and distinct DNA methylation pattern that controls the transcription of tissue-specific genes. Differentiated cells respond by generating a lasting and unique DNA methylation pattern that regulates the transcription of tissue-specific genes. (6). The study's goal of this study is to figure out the connection between VDR-gene promoter methylation in Iraqi GDM and its consequence on vitamin D levels.

## 2. Materials and methods

### 2.1 Study subjects:

In this case-control research conducted in a biotechnology research centre, a total of 50 patients with GDM (Group 1) and 30 healthy pregnant Control participants (Group 2) were obtained from Al-Mustansiriya National Diabetes Center and Al-Yarmouk Teaching Hospital/ obstetrics Dept. / Baghdad, From February 2021 to August 2021. Legal permission was arranged by hospital management, and the goal and benefits were explained to every volunteer and healthcare practitioner.

### 2.2 Bisulfite conversion:

Blood samples were extracted using Quick –g DNA blood minprep (Zymo,USA), extractExtracted DNA underwent bisulfite conversion according to the instructions for MethylEdge Bisulfite Conversion System (Promega; Madison, WI, USA). Briefly, 200-400 ng of each DNA sample was incubated for 8 min at 98°C and 60 min at 54°C with the Lightning Conversion reagent. Following the addition of the L-Desulphonation Buffer to the Zymo-Spin™ IC Column, the solution was kept at room temperature for another 20 min. Bisulfite-treated DNA was eluted in a total volume of 20 µl. All methylated and unmethylated CpG islands in the genome are converted by sodium bisulfite. It transforms unmethylated cytosines to uracil, which is found in single-stranded DNA, while leaving methylated cytosines alone. PCR Products were amplified according to the nucleic acid amplification protocol for all sets of primers, according to the instruction manual (AddBio) as follows: denaturation at 95°C for 5 min, 45 cycles at 94°C for 30 sec, 57°C for 45 sec, and 72°C for 45 sec. In all cases, a final extension at 72°C for 10 min was included.

### 2.3 Methylation detection and primer design

The methylation detection for the VDR promotor was done using the PCR method for patient's samples and control after bisulphate conversion treatment, PCR Products were amplified according to the nucleic acid amplification protocol for all sets of primers, according to the instruction manual (AddBio) as follows: denaturation at 95°C for 5 min, 45 cycles at 94°C for 30 sec, 57°C for 45 sec, and 72°C for 45 sec. In all cases, a final extension at 72°C for 10 min was included. Primers listed in table (2-1) were designed for a region in the promotor containing CpG island by using the online program [http://www.urogene.org/cgi-bin/methprimer\\_methprimer.cgi](http://www.urogene.org/cgi-bin/methprimer_methprimer.cgi) which determines the most candidate region for methylation in the promotor of the VDR gene.

Table (2-1): Sequence of Primers of methylation used in the present study

Primers	Methylated primers	Unmethylated Primers	Product size (bp)
Set 1	MF1:GTATTAATAGGGAGAGAAGGGCG	UMF1:GTTAGGTATTAATAGGGAGAGAA GGGT	122/ 128
	MR1: CAACTCCAAACTATATCCGACT	UMR1: TCAACTCCAAACTATATCCAACT	
Set 2	MF2:AAGTTAAGATGGTTGTAGCGTTA AC	UMF2:TAAAGATGGTTGTAGTGTAAAGG	195
	MR2:CGACGAATAAACAAACTATTCCG	UMR2:CCAACAAATAAACAAACTATTCC AC	

### 3. Results

#### 3.1 General study characteristics

In the present investigation, there were 30 healthy participants (group 1) and 50 patients with GDM (group 2). The GDM team comprised women with a mean age of  $31.92 \pm 0.94$  years. The healthy controls were aged with a mean of  $30.0 \pm 1.18$ .

#### 3.2 Methylation and amplified samples

Methylation was amplified in GDM samples and controls using a specific primer that resulted in a product with molecular weight (122bp for methylated pattern and 128 for UN-methylated product) figure (3-1).

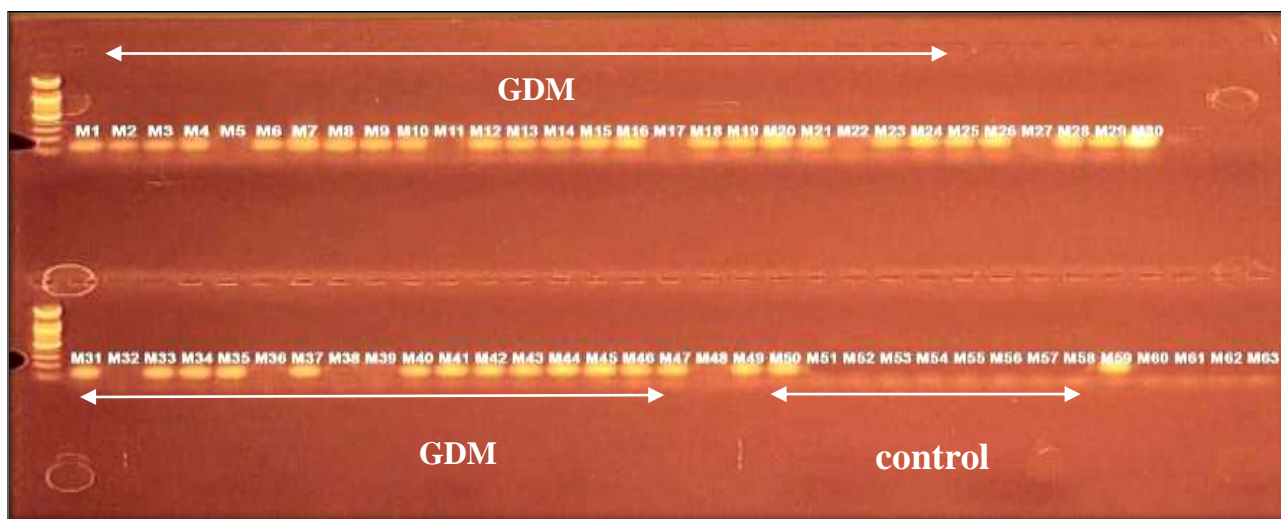


Figure (3-1): Illustrates Methylated fragments of DNA bands from GDM patients amplified on a 1% agarose gel at 5 volts/cm at 100 volts for 20 min.

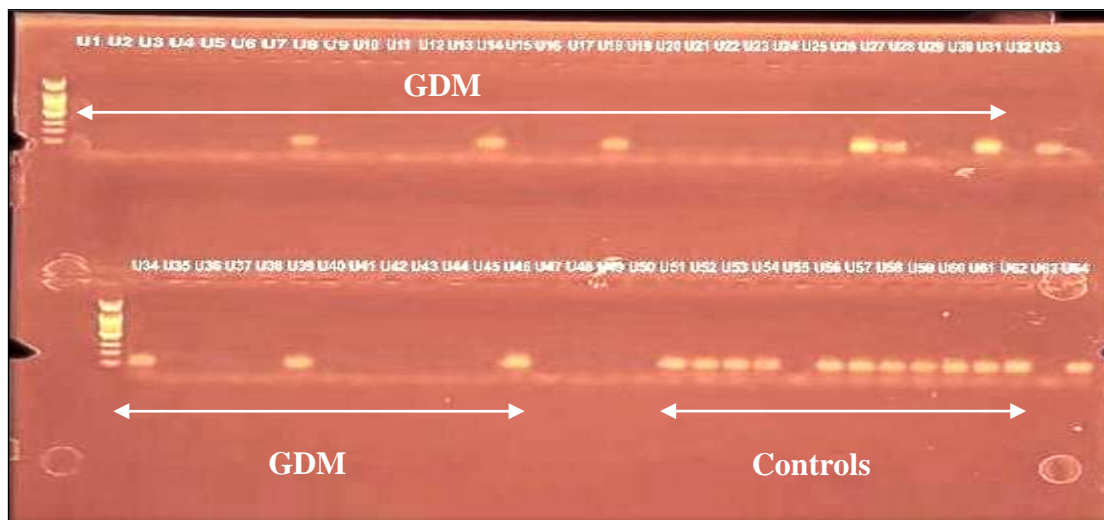


Figure (3-2): Illustrates UN-Methylated fragments of DNA bands from GDM patients amplified on a 1% agarose gel at 5 volts/cm at 100 volts for 20.

### 3.3 Results of methylation

Methylation was conducted for VDR gene promoter on all samples (GDM patients and controls) and the results indicated highly significant differences at p-value (0.00) for GDM patients and healthy pregnant controls. These results illustrated that observed findings (64.0%) of the GDM sample are methylated whereas (6.70%) of methylated controls were observed in the table (3-1). The Un-methylated GDM samples are observed (20.0%) compared to un methylated controls which about (86.70%) are observed un methylated results. Other partial methylated results were observed (16.0%) in GDM patients while appearing in (6.70%) in observed healthy controls (3-20). So the results of the methylation process of this study were incompatible with the Hardy-Weinberg equilibrium using the Chi-square test.

Table (3-1): Numbers and percentage frequencies of methylation genotype and their Hardy-Weinberg equilibrium (HWE) in patients and controls

Methylation	Patients group	Control group (%)	p-value	X <sup>2</sup>
	(%)			
Methylated	32 (64.0%)	2 (6.70%)	0.00	37.32
Partial methylated	8 (16.0%)	2(6.70%)		
Un-methylated	10 (20.0%)	26 (86.70%)		
Total	50 (100.0)	30 (100.0)		

The results of methylation effect on vitamin D3 expressed there were significant differences between methylated (25.74 ± 2.00) a percentage of 32(64.00%) of GDM samples compared with concentration (14.00±1.41) of methylated sample control at the percentage of observed results of methylation (1 (3.3%) at p-value (0.00).

UN-Methylated GDM samples 10(20.00%) represent the concentration of vitaminD3 (28.70 ± 3.22) while 26(86.70.0%) of UN- methylated control appeared (29.58±2.16) and this finding indicated that no significant differences between them at p-value (0.82).

However Partial methylated for GDM samples 8(16.00%) showed no significant differences at a concentration of vitamin D3 ( $27.90 \pm 8.20$ ) contrasting to control 2(6.70%) finding the result of vitamin D3 concentration ( $23.34 \pm 4.11$ ) at p (0.57).

**Table (3-2): association between serum of vitamin D levels and VDR gene Methylation process From GDM patients and controls**

Methylation	Vitamin D <sub>3</sub> mean $\pm$ SEM (mg/dl)		Probability
	Patients group	Control groups	
<b>Methylated</b>	<b>25.74 <math>\pm</math> 2.00</b>	<b>14.00<math>\pm</math>1.41</b>	<b>0.00*</b>
<b>Partial methylated</b>	<b>23.34 <math>\pm</math> 4.11</b>	<b>27.90<math>\pm</math> 8.20</b>	<b>0.63</b>
<b>Un-methylated</b>	<b>28.70 <math>\pm</math> 3.11</b>	<b>29.58<math>\pm</math>2.16</b>	<b>0.82</b>
	<b>0.16</b>		<b>0.72</b>

#### 4. Discussion

Vitamin D is a hormone-produced precursor. In addition to maintaining the balance of calcium and phosphorus metabolism, vitamin D has various non-classical effects, involving immunomodulatory and inflammatory responses, cell proliferation and differentiation control, and glycolipid metabolism. Epigenetic alteration of VDR signalling is one mechanism described for the reduced responsiveness to 1,25-D3 actions. It can also be due to an abnormal buildup of VDR-associated co-repressors, especially at the promoters of anti-proliferative target genes, or by the methylation of the promoters of important genes for the vitamin D system. (7).

It has been indicated that VDR gene polymorphisms increased the risk of diabetes mellitus (8) and GDM (9), suggesting the critical role of VDR in disease development. Furthermore, the study of gene methylation status, as a factor contributing to susceptibility to diseases such as cancer and diabetes, has been interested by many researchers (10). GDM has been suggested to occur due to the synergistic effect of both genetic factors and nutritional status. Moreover, Vitamin D deficiency has been shown to increase the risk of GDM (11).The current study is not agreed with Ponsonby et al., (2009) that established the VDR gene promoter was un-methylated in both GDM and those with no GDM (12) Awamleh et al., (2021) indicated the part DNAm plays in the placental and fetal reactions to hyperglycemia (13). However, grouping GDM samples for DNAm analysis according to therapy may make it easier to determine whether DNA methylation variations are due to adaptive responses or treatment effects in utero. Another study reported revealed in the case-cohort group, 18 of the 198 potential CpGs provide signs of interaction with 25(OH) D to modify the probability of developing breast cancer. (14).

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## إرتباط المثيلة الجينية بمستوى مستقبلات فيتامين (د) لدى عينة المرضى العراقيين بسكر الحمل

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### الخلاصة

**خلفية عن الموضوع:** داء سكر الحمل هو احد مضعفات الحمل المهمة التي تصاب به النساء الاتي لم يسبق لهن الاصابة بمرض السكري وارتفاع نسبة السكر بالدم المزمع اثناء الحمل حيث يحدث في معظم الحالات ارتفاع السكر في الدم بسبب ضعف تحمل الكلوكونز الناجم عن خلل وظيفي في خلايا البنكرياس مقابل وايضا نتيجة مقاومة الانسولين. مثيلة الحمض النووي هي عملية جينية تحدث في السايتوسين C5 لجينوم الثدييات وتتضمن اضافة مجموعة ميثيل لانتاج 5 ميثيل الستايتوسينز مثيلة الحمض النووي تنظم التعبير الجيني من خلال جذب البروتينات المشاركة في تنظيم الجينات او تثبيط عوامل النسخ من الارتباط بالحامض النووي. **الهدف من الدراسة:** تم تصميم هذه الدراسة للكشف عن العلاقة بين مثيلة موسس الجين وتأثيرها على مستوى فيتامين د لمرض سكر الحمل العراقيين. **طرق ومواد العمل:** تم جمع 50 عينة من مرضى سكر الحمل و30 عينة من الاصحاء الحوامل ثم عمل تحليل المثيلة بواسطة طريقة ال Bisulfate والبودئ الخاصة. **النتائج:** اظهرت نتائج المثيلة وتأثيرها على فيتامين د بوجود فروقات ذات دلالة احصائية كمقارنة بين المرضى بسكر الحمل والاصحاء اما نتائج غير المثل والمثل الجرئي فلم تظهر اي فروقات. **الاستنتاجات:** الحمل هو حالة من النشاط الايضي العالي والحفاظ على توازن الجلوكوز امر بالغ الاهمية من المحتمل ان تلعب جميع الجينات والجينات الاجينية دور في تطور مرض سكر الحمل، الفهم الاكبر لهذه العمليات ومشاركتها في داء سكر الحمل مطلوبة لغرض العلاجات المؤثرة.

الكلمات المفتاحية : سكر الحمل ، جين VDR وفيتامين د ، المثيلة الجينية.