

Vitamin D Status and its Receptor Genes *BsmI*, *FokI*, *Apal*, *TaqI* Polymorphism in Relation to Glucose Metabolism in Obese Iraqi Type 2 Diabetes Mellitus Patients

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Abstract

Background: Vitamin D receptor (VDR) gene polymorphisms are possibly involved in the development of type 2 diabetes mellitus (T2DM). However, the data to date have been inconclusive. Previous studies have suggested an influence of vitamin D receptor alleles on glucose metabolism and on susceptibility to type 2 diabetes mellitus in different ethnic populations through the action of vitamin D endocrine system which related with calcification and lipolysis, insulin secretion, and may be associated with many complicated disease including diabetes. To investigate the relationship between a single nucleotide polymorphism (SNP) of VDR gene and T2DM more studies had been done. However, different results have been found in different spots of the world. Therefore, more studies are needed to understand the variation in these results. This is the first study that shows the implication of the SNP of VDR gene in T2DM in Iraqi patients.

Objective: To assess the correlation between serum 25(OH)D₃ levels, and vitamin D receptor (VDR) polymorphisms (*FokI*, *BsmI*, *TaqI* and *Apal*), and glycemic control in obese T2DM Iraqi population, this study was performed.

Materials and methods: 200 clinically diagnosed T2DM patients, distributed into three subgroups according to therapeutic pattern and 75 healthy controls from the Iraqi population were recruited in this study. The association between the VDR gene SNPs and the T2DM was determined using Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique, and genotype and allele frequencies were calculated between the T2DM and control groups.

Results: No significant differences between mean age and the body mass index between both case and control groups were observed. According to current glycemic control consensus, results demonstrated only 20.5 percent of the T2DM patients met this target, which meant that 79.5 percent of the cases were suffering from poor glycemic control 25(OH)D₃ levels were significantly lower in the T2DM patients than in the control group, being 17.49 ± 1.12 ng/ml and 31.26 ± 1.25 ng/ml, in the patient and control groups, respectively ($p < 0.001$, Student's *t*-test). 25(OH)D₃ levels were found to be inversely associated with HbA_{1c} levels in the T2DM patients ($p < 0.001$, $r^2 = 0.058$). In the group of T2DM patients, 160 of 200 (80 percent) as opposed to 2 of 75 (2.6 percent) in the non-diabetic I group had lower, 25(OH)D₃ levels ≤ 20 ng/ml (chi-squared test, $p < 0.001$), while in the group of type 2 patients, 15 of 200 (7.5 percent) as opposed to 5 of 75 (6.6 percent) in the control group, had vitamin D insufficiency, 25(OH)D₃ levels > 20 < 30 ng/ml (chi-squared test, $p < 0.0001$). The gene polymorphism analysis for T2DM showed that genotype and allele frequencies for the VDR genes were in agreement with Hardy-Weinberg equilibrium in all cases.

Conclusion: VDR gene polymorphism analysis revealed that neither genotypes nor alleles of VDR *BsmI*, *Apal* showed a significant variation between T2DM patients and controls. In contrast, the FF genotype of VDR *FokI* and TT genotype of VDR *TaqI* showed a significant ($P < 0.0001$) increase in T2DM patients in comparison to controls. FF and TT homozygotes had significantly higher baseline fasting glucose and HOMA-IR levels than F allele carriers. In addition, data found significantly elevated interleukin IL-6, TNF- α , IL-1 β and decreased osteocalcin in association with the *TaqI* polymorphism.

Keywords: Vitamin D; Diabetes mellitus; Vitamin D; Receptor gene; Polymorphisms

Introduction

Diabetes Mellitus of type-2 is known to be one of main chronic diseases of related to non-communicable. Moreover, is there are any of the complications noticed, it be the pathway to immediate death in a nationwide space. This is one of the main reasons why the world is losing so many people with the complication of the disease. It can be said that, worldwide, more than 285 people in millions are suffering from diabetes. Most of the people are suffering from the type 2 of the diabetes according to the International Diabetes Federation, which can be abbreviated as IDF in the year, 2011. In the present time, the lack of Vitamin D is considered to be an issue that is related to the public health around the globe. By the year 2008, it was accounted that more

than a billion of the individuals around the world showed that they were suffering from deficient of Vitamin of type D. Also, there has been seen that the deficient caused abnormal metabolism in glucose along

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with Type 2 of diabetes. This has been clearly visible with the records that have been shown in the reports by the health organization [1,2]. Clinical reviews have demonstrated a constructive relationship between flowing vitamin D (25-hydroxyvitamin D; 25(OH)D) levels and insulin affectability, showing that vitamin D inadequacy may incline individuals to glucose narrow-mindedness, adjusted insulin emission and sort 2 diabetes [3]. This is done either through direct activity by means of vitamin D receptor (VDR) initiation or by implication by means of both calcemic hormones and irritation [4,5]. Chronic inflammation is often observed or seen in the people who are obese. Their body is associated with the building of the Resistance of insulin. This gives rise to the risk of growing a deficient [6] of diabetes of type 2. Hotamisligil is the first one who formed a link within the obesity, insulin along with inflammation [7]. It is quite evident that the activity of provocative pathways interferes with the metabolism which is normal and also causes disruption the proper signaling of the insulin. This increases the expression of the pro-inflammatory cytokines [8]. The cytokines have the target of attacking the receptors cell membranes, responding to the inflammatory resistance along with the exacerbating insulin resistance [9]. Another critical sub-atomic go between that connections pro-inflammatory cytokine to hindrance of insulin flagging is silencers of cytokine flagging (SOCS) 1 and 3, actuated by IL-6, which prompt ubiquitinylation and debasement of insulin receptor substrate (IRS) proteins [10]. The number of evidences that is increasing from time to time; clinical as well as studies from observation with systematic review states that the low-intensity and sub clinical inflammation reaction exists. This even precedes the T2DM of the situation [11-13].

The incendiary markers that have demonstrated the most grounded prescient limit in the advancement of T2DM are C-receptive protein (CRP) and IL-6 [11,14]. Epidemiological reviews have demonstrated that TNF- α , CRP, and IL-6 are emphatically related with BMI and muscle to fat quotient [15,16]. A few late human reviews have related vitamin D status with sort 2 DM improvement [13]. In spite of the fact that the mechanisms are essential the part of vitamin D as a hazard (Figure 8) a DM stays unexplained. It was demonstrated that 25(OH) D3 could be identified with the event of diminished insulin discharge and affectability [17] weight [18] diabetes, and hypertension [19]. Vitamin D receptor (VDR) is circulated in more than 38 tissues, where it apparently controls essential qualities identified with bone metabolism, oxidative harm, ceaseless ailments and irritation [20]. Vitamin D and its receptor complex assume a part in managing B-cell insulin emission and a few polymorphisms, for example, *BsmI*, *FokI*, *TaqI* and *Apal* have been portrayed in the VDR qualities that can modify the action of VDR protein [21]. All VDR polymorphisms are situated between exons 8 and nine aside from that *FokI* is in exon 2. It has been shown that some of these polymorphisms are related to sorting two diabetes mellitus, insulin discharge [22,23] and also to metabolic changes identified with stoutness [21]. Few reviews have inspected VDR quality polymorphisms for association with the danger of these scatters [21,24]. Human reviews researching the impact of vitamin D status with VDR hereditary SNPs on provocative biomarkers of subjects with or at high danger of creating sort two diabetes and its complexities are rare and have produced clashing outcomes. As far as anyone is concerned, no distributed information is accessible concerning the conceivable interaction amongst vitamin D and the insusceptible/fiery go-between in sort two diabetes mellitus in Iraq. To date, be that as it may, it stays to be elucidated whether the second-rate inflammation observed in type two diabetes mellitus may be impacted by the insusceptible properties of Vitamin D. Consequently, this review was intended to analyze the part of Vitamin D status in low-power constant aggravation and insulin

resistance in T2DM. Also, we proposed to explore the relationship of VDR quality polymorphisms (*BsmI* as a hazard consider) in DM parts Iraqi patients. It is likewise obscure whether vitamin D status is identified with lipids digestion, and whether the relationship between plasma vitamin D and insulin resistance and also insulin emission is as yet critical in existing T2DM. In this way, the global target of this paper has been revealed insight into these uncertainties in Iraqi individuals. The particular objectives have been: To gauge the pervasiveness of vitamin D inadequacy and the connection between vitamin D status with glycemic control in existing T2DM. In addition DNA genotyped for polymorphism of VDR gene at position sites of *FokI*, *BsmI* *Apal* and *TaqI* by PCR-RFLP to confirm its correlation with T2DM patients.

Materials and Methods

Participants

A case control study was undertaken by the Biotechnology department at College of Science, Baghdad University, between March 2015 and June 2016. The Scientific Ethical Committee of College of Science at Baghdad University also approved the study. The current study was carried out on some patients ($n= 200$) suffering from type-2 diabetes who attended outpatient clinic of the National Center for the treatment of diabetes and Research, College of Medicine at Al-Mustansiriyah University. There were three groups: 50 T2DM patients, newly diagnosed and using physical activity and lifestyle for treatment; 75 T2DM patients with oral anti-hyperglycemic drugs used as a treatment, and finally 75 T2DM patients with a combined treatment of oral anti-hyperglycemic drugs and insulin injections. The samples group comprised of 128 males and 72 females, aged from 34 to 63, with a mean value \pm S.D. of 47.79 ± 7.24 years, with a body mass index 29.2 ± 1.3 kg/m².

Management and screening of the chosen patients was done on the basis of the guidelines of American Diabetes Association. A total of 75 persons with sex and age-matched, obese, normal and healthy as the controls were then enrolled for this study. They were enrolled as chosen from the healthy staff of the stated institute and university with 32 females and 43 males. Their mean ages, in this case ranged between 37 to 61 years and with a mean value \pm S.D. of 47.96 ± 5.61 years. They were matched on socioeconomic background, BMI, age, sex and no evidence of DM was found. The patients were screened under the standard oral glucose tolerance test. Then questionnaire was formed on lifestyle, socio-demographic information, and geographical origin, past medical history, family history, existence of hypertension, type-2 diabetes and the treatment use. Anthropometric measurements such as body mass index, age, gender, weight, height, and systolic and diastolic blood pressure were undertaken for every volunteer.

Inclusion criteria

These patients were considered as cases with diabetic mellitus type 2 if they had the following criteria: fasting blood sugar more than 140 mg/dl post-prandial sugar more than 200 mg/dl, impaired OGGT and elevated HbA_{1c}.

Exclusion criteria

Participants were not included if they were found to have liver or kidney disease or extreme cardio-cerebral vascular disease or cancer. Pregnant participants were also excluded. In addition, participants with Diabetes type 1, with abnormal thyroid function, Vitamin D administration or with any osteoporosis. Patients who did not meet these criteria but who gave a history of T2DM were included in the

Polymorphism	Location in VDR gene	Restriction site	Digested Alleles	Primer Sequence	Annealing Temperature (°C)	Digestion Protocol	Amplicon Length (bp)	Restricted Fragments (bp)
BsmI	Intron-8	GAATGC (1/-1)↓	B, b	Forward: 5'-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3'	63	37°C overnight	825	6,50,175
				Forward: 5'-CAA CCA AGA CTA CAA GTA CCG CGT CAG TGA-3'				
FokI	Exon-2	GGATG (9/1)↓	F, f	Forward: 5'-AGC TGG CCC TGG CAC TGA CTC TGC TCT-3'	68	37°C overnight	265	196,69
				Reverse: 5'-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3'				
ApaI	Intron-8	GGGCC↓C	A, a	Forward: 5'-CAG AGC ATG GAC AGG GAG CAA-3'	62	37°C overnight	740	5,30,210
				Reverse: 5'-GCAACT CCT CAT GGC TGA GGT CTC-3'				
TaqI	Exon-9	T↓CGA	T,t	Forward: 5'-CAG AGC ATG GAC AGG GAG CAA-3'	62	55°C 4 h	740	PS+: 290,245,205 PS-: 495,245
				Reverse: 5'-GCAACT CCT CAT GGC TGA GGT CTC-3'				

Table 1: Characteristic features of the four studied polymorphisms in the vitamin D receptor gene using (*FokI*, *BsmI*, *TaqI* and *ApaI*).

study. The criteria of exclusion were: presence of persistent diseases that could possibly alter metabolism of vitamin D, intake of vitamin D supplements medications affecting bone metabolism and breastfeeding or pregnant women.

Blood samples collected from all participants after 12–14 h fasting was divided in two tubes either with EDTA for genotyping and HbA1c determination or without the anticoagulant for serum chemical analyses. Glycemic status, lipid profile, Fasting serum glucose (FBG) and insulin concentrations as well as serum lipid profile and were determined as previously described [3]. Insulin resistance was evaluated by quantitative insulin check index. Circulatory 25(OH) D and intact parathyroid hormone were determined using enzyme immunoassay (Germany). DNA was extracted from anticoagulated blood samples using by using wizard genomic DNA purification kit (Promega-USA) according to the manufacturer's protocol. The *BsmI*, *FokI*, *ApaI* and *TaqI* polymorphic sites for VDR gene were considered. Polymerase chain reaction (PCR) amplification was performed using 3 sets of primers VDR1 (for *BsmI*), VDR2 (for *FokI*), and VDR3 (for both *ApaI* and *TaqI*) as described according to Garcia D et al. using the thermo cycler. After initial denaturation for 5 min at 95°C, samples were subjected to 30 cycles of amplification, 25 s at 94°C, 20 s at the relevant primer pair annealing temperature and 20s at 72°C. The final step was a 5-min. hold at 72°C. Amplified DNA was digested overnight with suitable amounts of reference restriction enzymes (Fermentas) in the restriction protocol at 37C or 4 h at 55°C according to the manufacturer's instructions. Digestion products were electrophoresed on 2% agarose gel containing 0.4 mg/L ethidium bromide. The polymorphism was documented by photographing under UV illumination.

Polymorphism analysis

All data concerning the 4 restriction enzymes and their restriction patterns are summarized in Table 1. Aliquots of 0.1 U of *BsmI*, *FokI* and *ApaI* and 3U of *TaqI* restriction enzymes (Fermentas, Lithuania) and 2 μL buffer were added to 4 μL of the VDR PCR products. The alleles were designated as *B* (650 bp and 175 bp fragments) and *b* (825 bp fragment) for *BsmI*, *F* (196 bp and 69 bp fragments) and *f* (265 bp) for *FokI* and *A* (530 bp and 210 bp) and *a* (740 bp) for *ApaI*. *TaqI* has 2 binding sites on the PCR product, one product a 495 bp and a 245 bp fragment (*t*) and the other at the single nucleotide polymorphism site in the presence of which the 495 bp fragment will be cut into a smaller 290 bp piece and a 205 bp piece (*T*).

Statistical analysis

The Hardy Weinberg equilibrium (HWE) equation was utilized to calculate the expected genotype frequency. Allele frequencies of cytokine genes were calculated by direct gene counting methods, while a significant departure from Hardy-Weinberg (H-W) equilibrium was estimated using H-W calculator for two alleles. The Hardy Weinberg equilibrium is the predicted frequencies of genotypes if mating is non-assortative and hardly having mutations from one allele to another. When there are two alleles for a particular gene, A and B, and their respective population frequencies are p and q, then the expected frequencies of the genotypes AA, AB and BB are p², 2pq and q², respectively. Significant differences between the observed and expected frequencies were assessed by Pearson's Chi-square test.

The difference between the expected and observed genotypes was assessed by Chi square X2 test. The P-value<0.05 was measured statistically significant. The frequencies of the alleles and genotypes were evaluated mutually T2DM patient and controlling sets by the Chi square test when appropriate. The odds ratio (OR) was used to estimate the ratio of the risk of Vitamin D status among patient groups with various allele or genotypes compared with the control group. The odds ratio (OR) and 95 percent confidence interval (CI) were also estimated in order to test the relation between T2DM, and investigate means and standard deviations (SD) [25,26].

Results

The clinical characteristics of cases with T2DM and non-diabetic healthy group are summarized in Table 2. No significant differences in mean age and BMI was observed between both groups while patients with T2DM had a significant lower vitamin D level (26.07 ± 13.03 ng/ml vs. 30.28 ± 13.05 ng/ml, p<0.01in healthy control group. Hypo-vitaminosis D was quite prevalent in most Iraqi T2DM patients compared with the healthy non-diabetic group as showed in Table 3. 140 (70.0 percent) of the cases suffered from moderate vitamin D deficiency and 20 (10 percent) had severe deficiency as shown in the Table 2. 15 (7.5 percent) patients were also vitamin D insufficient, leaving only 25 (12.5%) of all the subjects remaining who had sufficient blood vitamin D. There were no significant differences in mean age or mean BMI between DM Type 2 cases and the control (p>0.05). Fasting blood insulin, fasting blood glucose glycosylated hemoglobin, HOMA index, serum triglyceride, serum interleukin- 6, interleukin 1-β, tumor

Parameter	Non-DM	T2DM Life style	T2DM Oral drug	T2DM Oral + Insulin
BMI (Kg/m ²)	29.6 ± 0.15	29.3 ± 0.15	29.5 ± 0.42	29.78 ± 0.34
Age (year)	57 ± 1.6	56 ± 1.5	58 ± 1.7	56 ± 1.9
FBS (mg/dl)	86.6 ± 0.62	166 ± 2.92**	190 ± 5.86**	179.2 ± 4.46**
HbA1c (%)	5.23 ± 0.08	7.12 ± 0.07**	8.04 ± 0.08**	7.66 ± 0.122**
DM duration, Yr	0	1.1 ± 0.2	5.3 ± 2.1**	6.2 ± 2.7**
Ionized Ca ²⁺ (mM)	1.82 ± 0.26	1.25 ± 0.12*	1.10 ± 0.07*	1.11 ± 0.06*
Phosphate (mg/dl)	3.12 ± 0.55	2.66 ± 0.3*	2.72 ± 0.2*	3.53 ± 0.4*
Leptin mg/dl	28.2 ± 0.35	11.8 ± 0.24*	16.6 ± 0.28*	21.7 ± 0.36*
Adiponectin (mg/dl)	12.25 ± 0.20	28.6 ± 0.35**	21.9 ± 0.24**	17.3 ± 0.03**
PTH (pg/ml)	18.35 ± 2.85	8.75 ± 0.09*	10.2 ± 0.12*	13.05 ± 0.20*
Apolipo protein (a)	132.3 ± 1.46	108 ± 0.8*	118.6 ± 0.47*	128.8 ± 0.58*
Apolipo protein (b)	81.3 ± 1.57	66.6 ± 0.57*	69.9 ± 0.39*	78.2 ± 0.56*
Vitamin D3 (ng/ml)	31.26 ± 1.25	21.9 ± 0.48*	21.1 ± 0.43**	17.49 ± 1.12**
Insulin (mu/mL)	11.9 ± 0.12	14.7 ± 0.33*	17.6 ± 0.36*	28.9 ± 0.36**
HOMA-IR	3.25 ± 0.05	10.61 ± 0.07	7.54 ± 0.053	9.73 ± 0.04
Cholesterol (mg/dl)	179 ± 10.6	188 ± 11.2	195 ± 10.9	200 ± 11.7
Triglycerides (mg/dl)	125 ± 8.7	150 ± 8.7*	166 ± 8.7*	187 ± 8.9*
HDL-Chol (mg/dl)	45 ± 3.4	43 ± 3.8	40 ± 3.1	37 ± 3.0
LDL-Chol (mg/dl)	112 ± 3.4	115 ± 8.9	120 ± 3.4	128 ± 3.4
IL-6 (pg/ml)	22 ± 2.8	27 ± 3.5*	30 ± 3.9*	34 ± 3.4*
TNF-alpha (pg/ml)	11 ± 1.4	14 ± 2.0*	18 ± 1.7**	22 ± 1.9**
IL—1B (ng/ml)	1.8 ± 0.4	2.2 ± 0.32*	2.7 ± 0.36*	3.1 ± 0.26*
hsCRP (mg/dl)	0.33 ± 0.12	0.66 ± 0.19	0.99 ± 0.27*	1.21 ± 0.28**
Osteocalcin (ng/ml)	34 ± 2.3	21 ± 3.1*	18 ± 2.56**	13 ± 2.46**

Table 2: Baseline demographic and biochemical indices of T2DM cases (DM with life style, DM treated with oral drugs and DM treated with combination of oral drug and insulin) and non-diabetic healthy subjects (M ± SE).

Vitamin D level	Number of DM	Number of control
<10 ng/ml severe deficiency	20	0
10-20 ng/ml moderate deficiency	140	2
20-30 ng/ml insufficient	15	5
>30 ng/ml sufficient	25	69
Total number of samples	200	75
Odd ratio	69	
95% CI	28.52-166.88	
Z statistics	9.3	
Significance level	<0.0001	

Table 3: Distribution of vitamin D status in diabetic and control patients.

necrotic factor (TNF-α), serum reactive protein and serum adiponectin concentrations all increased significantly in all diabetic subgroups compared with the non-diabetic control, while serum concentration of leptin, apolipoprotein A and osteocalcin decreased significantly in all the diabetic groups compared with the control. Serum-ionized calcium and lipid profile decreased but not significantly in TC, TG, and LDL-C. All serum vitamin D levels were significantly lower in individuals with diabetic type 2 when compared to the controls as seen in Tables 2 and 3. There is no large scaled epidemiology study evaluating vitamin D deficiency in T2DM; however, several studies of different races have reported a high prevalence of hypo-vitaminosis D in patients with T2DM [27,28]. The distribution of vitamin D status in this study sample is very much in line with the study of 29 which found up to 98 percent of the subjects had vitamin D insufficiency or deficiency. Serum vitamin D concentrations in this study are significantly negatively associated with age as expected ($r=-0.54$ $P<0.001$). The main resource of vitamin D in our body is sunlight exposure. The elderly usually has decreased outdoor activities, due to the limitation of their self-care ability.

This grim situation and the high prevalence of T2DM diabetes have driven a search for potentially modifiable environmental factors, besides the application of anti-diabetic drugs and modification of life

style, in attempting to better manage diabetes. Basic science studies have revealed the plausible effects of vitamin D on the development of diabetes. Coupled with the easy administration and relatively economical price, vitamin D is an ideal candidate for modulating the early development of T2DM. Considering the high prevalence of vitamin D deficiency in established T2DM patients, if vitamin D is still significantly associated with glycemic control, vitamin D as an agent in diabetic management is rather appealing. Data of the present study showed that vitamin D status was significantly associated with HbA1c after adjusting for sex, age, BMI, and duration of diabetes. Data of this work correlates with another study in South Korea which explained the negative relationship between vitamin D status and glycemic control in existing T2DM, which reached a similar conclusion [29]. In addition, evidence from a number of studies has suggested a relationship between vitamin D and micro-vascular complications of T2DM. Moreover, vitamin D has been found to be inversely associated with cardiovascular events and impaired renal function in patients with T2DM [30,31].

The correlation between fasting blood glucose (FBG) and vitamin D status has hardly ever been estimated. Our data also showed a trend of negative correlation with vitamin D. When stratifying patients by BMI, FBG showed a significant inverse relationship with vitamin D, with a standard coefficient beta= - 0.066, and p-value<0.01. However, FBG is easily influenced by diet, activities, and anti-diabetic drug, resulting in its frequently fluctuations and nominal value in diabetes management.

Amplification of the VDR gene

Polymerase chain reactions were performed for the amplification of the VDR gene in all diabetic and non-diabetic groups. Agarose gel electrophoresis was used to confirm this amplification for each exon and intron following optimization experiment when sharp, single bands with the accurate molecular sizes were obtained for each exon and intron. The VDR gene was amplified using specific primers for the detection of each *FokI, BsmI, Taq I* and *Apal* SNPs. The balance

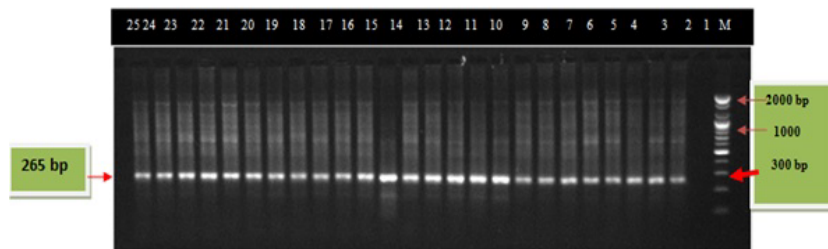


Figure 1: Agarose gel electrophoresis of PCR reaction for *VDR* gene SNP *Fok I* with gene products size (265) bp and the ladder marker were resolved on 1.5 percent agarose gel. The electrophoresis was undertaken at 70 V for 2 h and the gel was viewed under gel documentation – A UV trans-illuminator - after having been stained with ethidium bromide. M: Marker, Lane 1: Negative control, Lanes 2-25: Patients sample.

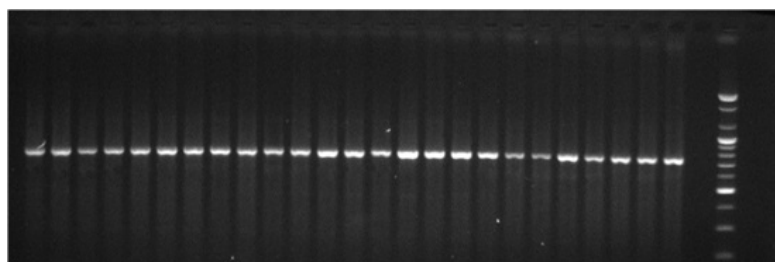


Figure 2: Agarose gel electrophoresis of PCR reaction for *VDR* gene SNP *Apa1I* with gene products size (740) bp and the ladder marker were resolved on 1.5 percent agarose gel. The electrophoresis was undertaken at 70 V for 2 h and gel viewed under UV trans-illuminator after staining it with ethidium bromide stain. M: Marker, Lane 1: Negative control, Lane 2-25: Patient samples.

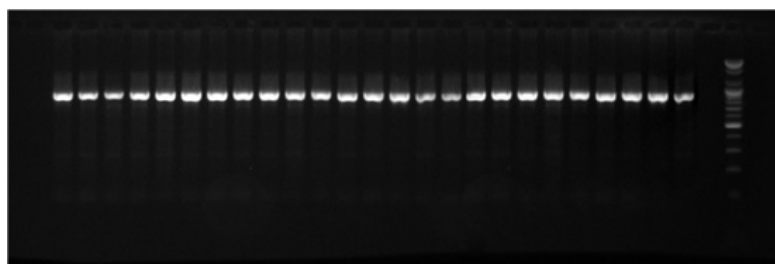


Figure 3: Agarose gel electrophoresis of PCR reaction for *VDR* gene SNP *BsmI* with gene products size (825) bp and the ladder marker were resolved on 1.5 percent agarose gel. The electrophoresis was undertaken at 70 V for 2 h and gel viewed under UV trans-illuminator after staining it with ethidium bromide stain. M: Marker, Lane 1: Negative control, Lanes 2-25: Patient samples.

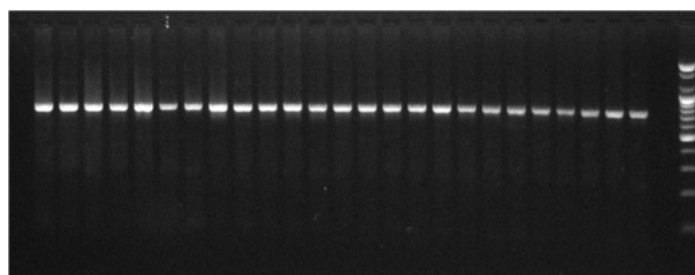


Figure 4: Agarose gel electrophoresis of PCR reaction for *VDR* gene SNP *Taq I* with gene products size (740) bp and the ladder marker were resolved on 1.5 percent agarose gel. The electrophoresis was undertaken at 70 V for 2 h and gel viewed under UV trans-illuminator after staining it with ethidium bromide stain. M: Marker, Lane 1: Negative control, Lanes 2-25: DM cases.

was usually achieved between reaction components to reduce the non-specific amplification or to enhance the desired DNA product yield. For this reason, the PCR condition was optimized before starting. In the PCR experiments the optimization of PCR conditions that include annealing temperature and annealing time that was performed in each PCR experiment. In the present study, the optimum annealing temperature for *VDR* primers was 68°C for 45 sec. The reaction components were

kept at the same concentrations. PCR negative control was performed in each experiment to determine any possible contamination and all negative control samples appeared as empty gel lanes in all experiments. The following figures demonstrate the amplified exons of *VDR* gene which are: exon 2 (Figure 1), exon 9 (Figure 2) and intron 8 (Figures 3 and 4). Amplification was achieved for all patients and controls. Genotypes for these polymorphisms are designated by the first letter of

the name of the enzyme. A capital letter indicates the absence of the cut site, whereas a lower-case letter indicates its presence.

PCR- RFLP genotyping of VDR gene

The results of PCR-RFLP analysis of the *FokI* of the *VDR* gene for T2DM and non-diabetic control subjects are shown in Figure 5 and Tables 4 and 5 which are summarized as the wild type homozygote

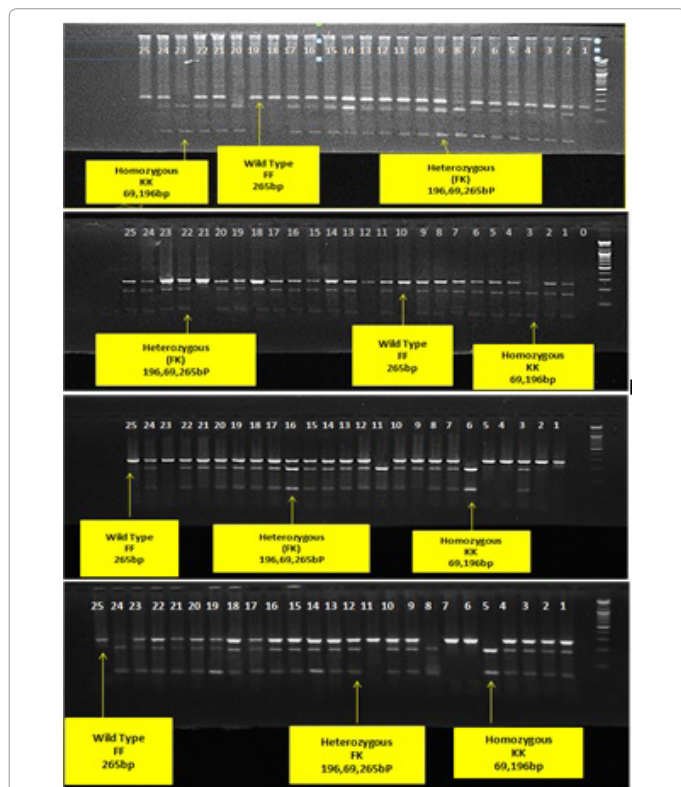


Figure 5: PCR-RFLP analysis of the *VDR* gene by the *FokI* in the type 2 diabetes mellitus patients and healthy subjects: The wild type homozygote (FF) showed one band (270 bp); heterozygote (Ff) showed three bands (265 bp, 196 bp, 69 bp). The product was electrophoresed on 2 percent agarose gel at 90 volt for 1 hour, stained with ethidium bromide, then visualized under U.V light. (A) refers to non-diabetic (B) Diabetic life style (C) Diabetic treated with oral hypoglycemic drug (D) Diabetic treated with oral drug and insulin injection. M: Marker (100-2000 bp).

(FF), heterozygote (Ff) and mutant homozygote (ff) showed one band (265 bp), three bands (265 bp,169 bp and 96 bp) two bands (169,96) bp respectively. The genetic polymorphism of *VDR* was determined at *FokI* site, which were presented with three genotypes (FF, Ff, ff for *FokI* in T2DM patients and controls. The genotype FF of *VDR* gene at *FokI* position demonstrated a significant ($P=0.0001$) increased percentage frequency in T2DM patients (70.5 percent) compared to controls (46.6 percent). The F allele was increased (80.25 vs. 56 percent), while f allele was increased in non-diabetic (44 vs. 19.75 percent) in the T2DM patients) and this increase was highly significant ($P<0.0001$).

The results of PCR-RFLP analysis of the *BsmI* of the *VDR* gene in T2DM are shown in Figure 6 and Table 6 which summarized as three genotypes, the wild type homozygote (BB), heterozygote (Bb) and mutant homozygote (bb) showed one band (825 bp), three bands (825 bp,650 bp and 175 bp) two bands (650 bp and 175 bp) respectively. The genetic polymorphism of *VDR* was determined at *BsmI* were presented with three genotypes (BB, Bb, bb) for *BsmI* in T2DM patients and controls. For *VDR BsmI*, the BB genotype frequencies were similar in the T2DM patients than the controls (35 percent vs. 32 percent, $P>0.05$). On the other hand, the Bb genotype frequencies were lower in the T2DM patients compared with the controls (61 percent vs. 66.6 percent, and the difference is not significant ($P>0.05$). Both B and b alleles frequency have the same value in both T2DM patients and controls, that B allele frequency in T2DM patients is 65.5 percent vs. 65.3 percent in the controls) and the b allele frequency have a similar percentage 34.5 vs. 34.6 percent in T2DM patients and controls respectively, as shown in Table 5.

On the other hand, the results of PCR- RFLP analysis of the *TaqI* digestion of the *VDR* gene in T2DM patients and controls are shown in Figure 7 and Table 6 which can be summarized into three genotypes: the wild type homozygote (TT) showed two band (495 bp and 245 bp); heterozygote (Tt) showed four bands (495 bp, 290 bp, 245 bp, 205 bp). Homozygote (tt) showed three bands (290 bp, 245 bp, 205 bp). *TaqI* has 2 binding sites on the PCR product, one product a 495 bp and a 245 bp fragment (t) and the other at the single nucleotide polymorphism site in the presence of which the fragment 495 bp will cut into a smaller 290 bp piece and a 205 bp piece (T). For *VDR* digested by *TaqI*, the results show the TT genotype frequencies were significantly higher in the T2DM patients than the controls (39 percent vs. 20 percent, $P<0.0034$), while the Tt genotype frequencies were lower in the T2DM patients than controls and the difference was not significant (47.5 percent vs.

Genotype or Allele	T2	DM Patients (No=200)		Controls (No=75)		OR*	95% CI	P value*	Z Statistics
	No.	%	No.	%					
FF	141	70.5	35	46.6	2.73	1.5820 to 4.7152	<0.0001	3.6	
Ff	39	19.5	14	18.7	1.05	0.5358 to 2.07	0.87	0.156	
ff	20	10	26	34.7	0.21	0.1079 to 0.4064	<0.0001	4.622	
F	321	80.25	84	56	3.19	2.12 to 4.78	<0.0001	5.6	
f	79	19.75	66	44	0.31	0.2 to 0.46	<0.0001	5.6	

Table 4: Observed numbers and percentage frequencies of *VDR* genotype and alleles at *FokI* position in T2DM patients and control.

Genotype or Allele	T2	DM Patients (No=200)		Controls (No=75)		OR*	95% CI	P value*	Z Statistics
	No.	%	No.	%					
BB	70	70	24	32	0.61	0.359 to 1.054	<0.077	1.76	
Bb	122	122	50	66.6	0.78	0.4477 to 1.36	0.3878	0.864	
bb	8	8	1	1.3	3.08	0.379 to 25.08	0.2924	1.053	
B	262	262	98	65.3	1.00	0.6790 to 1.49	0.9708	0.037	
b	138	138	52	34.6	0.9	0.6691 to 1.47	0.970	0.037	

Table 5: Observed numbers and percentage frequencies of *VDR* genotype and alleles at *BsmI* position in T2DM patients and control.

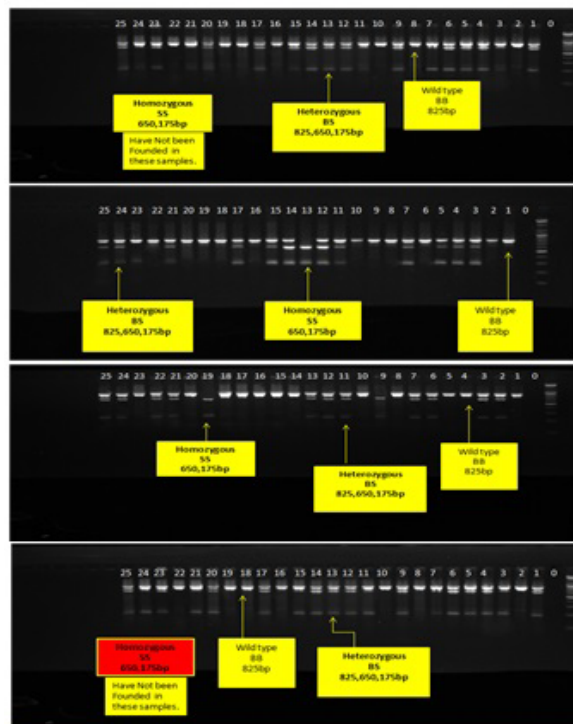


Figure 6: PCR -RFLP analysis of the VDR gene by the Bsm1 in the type 2 diabetes mellitus patients and healthy subjects: The wild type homozygote (BB) showed one band (825 bp); heterozygote (Bb) showed three bands (825 bp, 650 bp, 175 bp). The product was electrophoresed on 2 percent agarose gel at 90 volt for 1 hour, stained with ethidium bromide, then visualized under U.V light. (A) refers to non-diabetic (B) Diabetic life style (C) Diabetic treated with oral hypoglycemic drug (D) Diabetic treated with oral drug and insulin injection. M: marker (100-2000 bp).

Genotype or Allele	T2	DM Patients (No=200)		Controls (No=75)		OR*	95% CI	P value*	Z Statistics
	No.	%	No.	%					
TT	78	39	15	20	2.55	1.3578 to 4.81	0.0034	2.9	
Tt	95	47.5	44	58.66	0.637	0.3726 to 1.0	0.1002	1.644	
tt	27	13.5	16	21.33	0.575	0.2900 to 1.1	0.1141	1.58	
T	251	62.7	74	49.3	1.730	1.1845 to 2.5	0.0046	2.83	
t	149	37.3	76	50.7	0.578	0.3957 to 0.8	0.0046	2.836	

Table 6: Observed numbers and percentage frequencies of VDR genotype and alleles at *TaqI* position in T2DM patients and control.

58.6 percent, $P > 0.05$). Both T and t alleles frequency are significantly different between T2DM patients, compared with controls ($P < 0.004$).

A significant association between the attendance of a VDR *FokI* F allele and T2DM had been observed, while the VDR *FokI* f allele was more frequent among the control individuals. These finding suggest a protective role for the f allele in contrast to the role of F allele which seems to be a predisposing factor to T2DM in the Iraqi population. In addition, the results seem to reinforce the association of the FF genotype with the susceptibility to the T2DM. On the other hand, the present study did not find any significant differences in VDR gene polymorphism at position *BsmI* and *Apal* between the T2DM patients and the controls, an observation that corroborated with a meta-analysis study by Mackawy and Badawi [32] in which the FF genotype association and the *BsmI* genotypes and allele non-association with T2DM was clearly obtained. Results of this work are inconsistent with Errouagulet [33] who observed that the ff allele was associated with T2DM in Morocco population. However, further studies failed to show an association between this polymorphism and T2DM in a Polish and an Asian meta-analysis study [34-36].

Another new finding in this work revealed a significant association

between the presence of higher TT genotypes distribution and higher T allele frequency and T2DM, while tt genotypes distribution and t allele frequency are more frequent among control individuals. These finding suggest a protective role for the t allele in contrast to the role of T allele which seems to be a predisposing factor to T2DM in Iraqi population for the first time. In addition, the results seem to reinforce the association of the TT genotype with the susceptibility to the T2DM in addition to FF genotype yielded by *FokI* digestion of VDR gene.

As per the outcomes on allele frequencies and genotype conveyances of the VDR quality *TaqI* and *FokI* polymorphisms in patients with T2DM, information in this work concur well with past reviews which announced that allele frequencies and genotype dispersions of the VDR quality *FokI* polymorphism are essentially unique in T2DM patients, for example, different infections give points of interest. A few polymorphisms, for example, *BsmI* and *FokI*, have been depicted in the VDR qualities that can modify the movement of VDR protein as reported by Filus et al. [18]. All VDR polymorphisms are situated between exons 8 and 9 aside from that *FokI* is in exon 2 [19]. Nonetheless, the hereditary foundation of the illness still stays hazy. Some of these polymorphisms have been appeared to be related with sort 2 diabetes mellitus, insulin emission²⁰, and stoutness related

metabolic changes as reported by Filus et al. While the connection between vitamin D inadequacy and segments of diabetic difficulties has beforehand been illustrated [21-23], little research has been directed to date into VDR quality polymorphisms for relationship with the dangers [37,38].

Then again, the Hardy-Weinberg Equilibrium (HWE) condition examination is performed by ascertaining the allele frequencies and the subsequent expected frequencies of the genotype in view of these. On the off chance that the watched frequencies of genotype are near the normal genotype frequencies figured from the watched allele frequencies, then the populace is in Hardy-Weinberg Equilibrium and allele blends are probably going to be free of each other. While testing for Hardy-Weinberg (H-W) balance uncovered that T2DM patients demonstrated a critical variety in the circulation of VDR *FokI* genotypes ($P < 0.0001$) this was seen due to differences between the watched and expected frequencies of F/F, F/f and f/f genotypes. Besides, critical contrasts were seen in the control test, in which they and expected genotype frequencies ($P < 0.001$), as show in Table 7.

Correlation of VDR (*FokI*, *BsmI*, *TaqI* and *Apa I*) polymorphisms with metabolic and biochemical, immunological markers

The allele and genotype frequency distribution of VDR (*FokI*, *BsmI*, *TaqI* and *Apa I* genes among subgroups of T2DM patients according to therapeutic regimes and controls demonstrated significant differences between T2DM patients treated with a combination of oral hypoglycemic drugs and insulin injection in comparison with T2DM patients treated with only oral hypoglycemic drugs and only regarding the genotype and allele distributions of the VDR *FokI* and *TaqI* polymorphism. The FF genotype for the VDR (*FokI*) was significantly more frequent in T2DM patients treated with combination oral hypoglycemic drugs and insulin injection than in T2DM treated oral drugs only and controls ($X^2=5.61$, $P < 0.05$ and $X^2=15.13$, $P < 0.0001$) respectively. Otherwise results may not find a significant association between T2DM patients treated with combination oral hypoglycemic drugs and insulin injection with T2DM patients treated oral hypoglycemic drugs or lifestyle group compared with non-diabetic controls regarding the VDR (*BsmI* and *ApaI*) polymorphism genotype and allele distributions ($X^2=4.92$, $P > 0.05$, $X^2=3.13$, $P > 0.05$, $X^2=3.38$, $P > 0.05$) respectively.

The F allele is more frequent in the T2DM patients treated with combination oral hypoglycemic drugs and insulin injection, than in T2DM patients treated only oral hypoglycemic drug, as contrasted to the power group ($p < 0.001$), odds ratio (OR) and 95 percent CI for the F allele of VDR (*FokI*)=2.30 (1.37-3.86). On the other hand, results showed a significant difference in T allele frequency between T2DM patients treated with combination oral hypoglycemic drugs and insulin injection than in T2DM patients treated only oral hypoglycemic drug only group as compared to control group ($p < 0.001$ and control groups ($P < 0.001$). Furthermore, we additionally thought about the VDR (*FokI*, *BsmI*, *TaqI* and *ApaI*) genotypes with various clinical parameters of every single contemplated gathering.

Results from Table (Data not shown) present the sharing of all scientific variables according to the VDR genotypes experiential in the control subjects. No alliance between VDR *FokI*, *BsmI*, *TaqI* and *ApaI* polymorphisms is noticed with all clinical variables, Vitamin D and IL-6 levels. Lipid profile exhibited significant associations with *FokI* and *TaqI* only in contrast of *BsmI* and *ApaI* VDR polymorphism. Then, the same analyses were performed for all the subgroup; T2DM patients according to the therapeutic regimes observed higher levels of IL-6, TC, TG, and LDL-C with lower HDL-C and vitamin D levels were seen in FF genotype than ff genotype carriers of the VDR (*FokI*) polymorphism respectively. While BB homozygous for the *BsmI* VDR variation is not related with lower vitamin D values than found in others. Furthermore, the estimations of FPI, FPG, HbA1c, BMI are comparative among all members, autonomously of nearness or nonattendance of both VDR *FokI*, *BsmI* *TaqI* and *Apa I* polymorphisms.

No relationship between VDR *BsmI* polymorphism was observed with every clinical variable part contemplated, or other biochemical parameters with the exception of vitamin D serum levels which

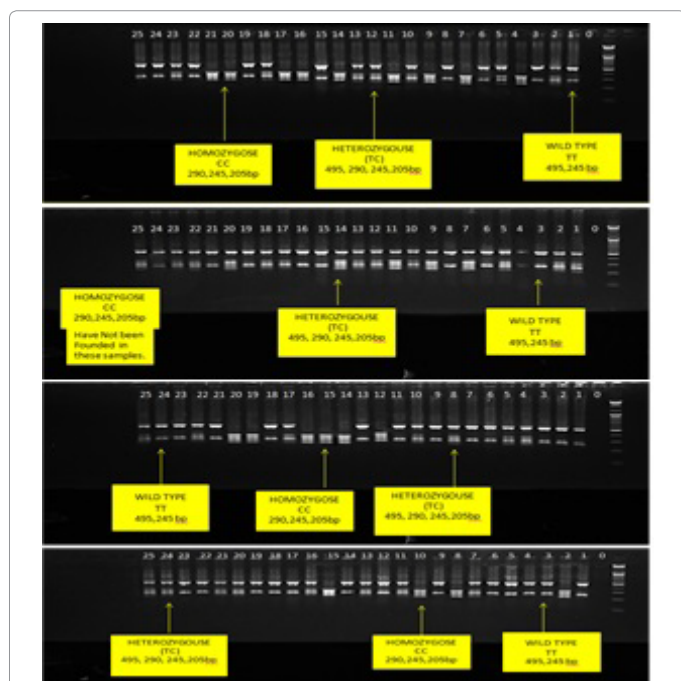


Figure 7: PCR-RFLP analysis of the VDR gene by the Taq1 in the type 2 diabetes mellitus patients and healthy subjects: The wild type homozygote (TT) showed two band (495 bp and 245 bp); heterozygote (Tt) showed four bands (495 bp, 290 bp, 245 bp, 205 bp). Homozygote (tt) showed three bands (290 bp, 245 bp, 205 bp). The product was electrophoresed on 2 percent agarose gel at 90 volt for 1 hour, stained with ethidium bromide, then visualized under U.V light. (A) refers to non-diabetic (B) Diabetic life style (C) Diabetic treated with oral hypoglycemic drug (D) Diabetic treated with Oral drug and insulin injection. M: Marker (100-2000 bp).

Genotype or Allele	T2	DM Patients (No=200)		Controls (No=75)		OR*	95% CI	P value*	Z Statistics
	No.	%	No.	%					
AA	59	29.5	26	34.6	0.7886	0.4485 to 1.3866	0.825	0.4095	
Aa	114	57	40	53.3	1.1599	0.6806 to 1.9766	0.545	0.5855	
aa	27	13.5	9	12	1.1445	0.5112 to 2.5623	0.328	0.7427	
A	232	58	92	61.3	0.8706	0.5930 to 1.2781	0.4793	0.707	
a	168	42	58	38.7	1.1486	0.7824 to 1.6863	0.4793	0.707	

Table 7: Observed numbers and percentage frequencies of VDR genotype and alleles at patients and control.

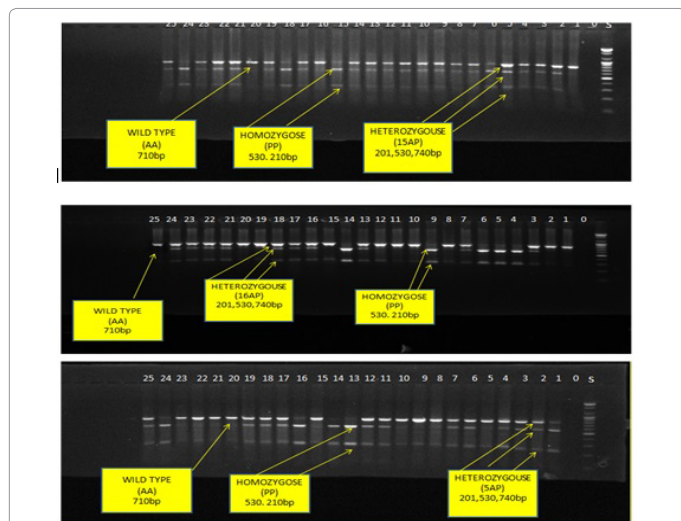


Figure 8: Presented the results of PCR- RFLP analysis at the *Apal* of the *VDR* gene > there are three genotypes the wild type homozygote (AA), heterozygote (Aa) and homozygote (aa) give one band (740) bp, three bands (740 bp, 530 bp, 210 bp) and two bands (530 bp and 210 bp) respectively. The genetic polymorphism of the *VDR* gene are presented at the *Apal* site, with three genotypes (AA, Aa, aa) for *TaqI* in all T2DM patients and controls. Results showed the AA genotype frequencies were lower in the T2DM patients than the controls (29.5 percent vs. 34.7 percent, $P=0.82$) while the Aa genotype frequencies were higher in the patients than controls but the difference is not significant (57 percent vs. 53.3 percent respectively, $P=0.58$). Both A and a alleles' frequency were not significantly different between T2DM patients compared with the controls ($P<0.05$), and A allele frequency in T2DM patients is 61.3 percent vs. 58 percent in the control group while the a allele frequency is 38.7 in T2DM patients vs. 42% in non-diabetic group as shown in Table 7.

demonstrated lower huge values in bb transporters. In any case, people conveying the mutant latent homozygous FF genotype of the *VDR FokI* polymorphism introduced fundamentally higher insulin levels, insulin resistance file (HOMA-IR), IL-6 serum levels, and BMI than people with the homozygous FF genotype ($P<0.001$). Also, the nearness of FF genotype of *VDR FokI* polymorphism is related with diminished vitamin D concentrations compared to Ff and ff genotypes ($P<0.001$).

In a trial to investigate the functional aspects of the four *VDR* polymorphisms, we measured several clinical and metabolic parameters about the T2DM suffer as well as controls included the purpose. The findings of present study revealed that the *VDR* gene polymorphisms might power the harshness of diabetic complications chaos. Otherwise, we could not discover momentous relationship stuck between *Apal* as well as *BsmI* *VDR* and the components of glycemic control in type 2 diabetic Iraqi patients except for vitamin D serum levels. In the present study, lipid profile parameters showed significant association with *VDR* 2228570 C N T (*FokI*) polymorphism in T2DM patients treated with a combination of oral and insulin injection, in addition, there were higher TC, TG, LDL-C and lower HDL levels in Ff and FF genotype carriers as well lower vitamin D levels in homozygous recessive *VDR* 2228570 CNT (*FokI*) and *VDR TaqI* [39-41].

Conclusion

In diabetic patients, we noticed significant associations between *VDR* 2228570 C N T (*FokI*) polymorphism as well as each of the insulin levels, insulin resistance (HOMA-IR), IL-6 levels, and BMI. Vitamin D levels were lower in homozygous recessive *VDR* 2228570 CNT (*FokI*) and *VDR* 1544410 ANG (*BsmI*). Moreover, though glycaemia is a major characteristic of T2DM, no affiliation was found between fasting

plasma glucose levels and *VDR* quality polymorphisms assessed in this review. Similarly, Malecki et al. and colleagues have explored *BsmI*, *TaqI*, and *FokI* *VDR* quality polymorphisms in 548 Polish people with and without T2DM and did not discover any relationship of glycaemia with any of the *VDR* quality polymorphisms either.

It is evident that hypo vitaminism D has prevailed in Iraqi population with T2DM. Alterations in vitamin D action could be lead to may affect Insulin-sensitivity, β -Cell action or both. Moreover, Data of this study demonstrated association between *VDR FokI* and *Taq-I* gene polymorphisms and susceptibility to T2DM in the Iraqi population in contrast to *VDR BsmI* and *Apa I* which showed no relationship. The possible action of vitamin D in the pathogenesis of T2DM is far from being completely understood. Additionally, further knowledge on this issue may identify new candidate targets in the treatment and prevention of the disease. Therefore, further investigations on this issue are warranted

Conflict of Interest

All these authors declare that they have no conflict of interest.

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