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Association between vitamin D receptor gene polymorphism and osteoporotic fractures among type II diabetic Egyptian females

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Abstract: The predominant role of the biologically active vitamin D is to regulate calcium homeostasis through increasing bone mineralization and intestinal calcium absorption. Moreover, vitamin D is found to be related to glucose metabolism, therefore vitamin D deficiency and vitamin D receptor (VDR) gene polymorphism may provoke type 2 diabetes mellitus (T2DM) and osteoporotic fractures as well. This case control study was conducted on 90 premenopausal females. Serum vitamin D_2, total calcium, albumin, ionized calcium and fasting plasma glucose were assayed. VDR gene TaqI single nucleotide polymorphism was assessed using polymerase chain reaction (PCR) using DNA isolated from the collected whole blood. Results showed that hyperglycemia, hypovitaminosis D_2 and low level of total calcium were observed in secondary T2DM osteoporotic fractures. G allele was significantly increased in the control group compared to patients with secondary T2DM osteoporotic fractures. Accordingly, this study concludes that the recessive G allele has a protective role against osteoporotic fractures in T2DM females.

Keywords: Osteoporosis; type 2 diabetes mellitus; vitamin D deficiency; single nucleotide polymorphism; premenopausal women.

1. INTRODUCTION

Diabetes mellitus is one of the chronic metabolic diseases that is described by hyperglycemia and classified according to the underlying insulin status either absence of insulin or insufficiency into type I diabetes mellitus (T1DM) or type 2 diabetes mellitus (T2DM), respectively [(1,2)].

Type 2 diabetes mellitus is the most prevalent chronic disease worldwide. Egypt, for example is ranked the seventh in the prevalence of T2DM with 8.9 million diabetic patients in 2019. However, 16.9 million of Egyptians are expected to be diabetic by 2045 [(3)].

Moreover, diabetes mellitus especially T2DM is linked with serious microvascular and macrovascular complications induced by chronic hyperglycemia [(4)]. However, a linkage has been found between T2DM and osteoporosis [(5)].

Osteoporosis is a chronic disorder in which prolonged bone resorption causes a progressive decline in bone mineral density (BMD), resulting in bone fragility and osteoporotic fractures. It is categorized based on the underlying cause into primary osteoporosis and secondary osteoporosis. Aged male osteoporosis and postmenopausal osteoporosis are examples of primary osteoporosis. Secondary osteoporosis is induced by some medications, or medical conditions such as diabetes mellitus [(6)].

The prevalence of osteoporosis in diabetes mellitus increases noticeably as 43.8% of T2DM patients complicate osteoporosis [(7)].

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In the last decades, vitamin D has gained a great interest due to its various skeletal and extra-skeletal functions [8]. Vitamin D is the sunshine vitamin that deploys its genomic function through vitamin D receptor (VDR) binding. Vitamin D and VDR present in different tissues, therefore, vitamin D deficiency and VDR gene polymorphism are associated with different diseases [9].

Beside its main role in the maintenance of calcium homeostasis, vitamin D is found to have a vital role in insulin secretion [10,11]. It affects pancreatic β-cells through direct stimulation of insulin secretion or indirect through its regulation of the intracellular and extracellular calcium. Therefore, vitamin D insufficiency may influence insulin sensitivity resulting in insulin resistance and development of T2DM [12].

Vitamin D receptor gene is placed on 12q13.11. FokI, BsmI, TaqI, ApaI are the main polymorphs present in the VDR gene [13]. The TaqI rs731236 A>G is the single nucleotide polymorphism (SNP) of interest in this study.

Interestingly, VDR TaqI genotypes had not any association with T2DM patients in Egyptian population [14]. Therefore, this study aims to examine the influence of VDR TaqI rs731236 on osteoporotic fractures as a complication of T2DM in Egyptian females.

2. METHODS

2.1 Subjects

This case control study was executed on 90 pre-menopausal females with age range (35- 42) years, including 45 non-vertebral secondary T2DM osteoporotic fractured patients and 45 healthy controls matched with the patients in the ethnic and demographic characteristics. All participants signed a written consent as well.

Bone mineral density was assessed in all patients using dual energy X-ray absorptiometry (DEXA) scan.

Patients with any other diabetic complication, renal or hepatic dysfunctions or other musculoskeletal problems were excluded. Additionally, patients who were on medications that may modify vitamin D level or affect its metabolism were excluded.

2.2 Methods

2.2.1 Sample collection and biochemical analysis

Five mL of blood were collected and aliquoted into EDTA, fluoride and gel vacutainer tubes for DNA extraction, glucose determination and serum collection, respectively.

For the determination of vitamin D_2, total calcium, and albumin; serum samples were aliquoted and stored at -20°C till the analysis time. Total calcium was measured using a spectrum diagnostics colorimetric kit (catalog no. 226 002, Egypt), and albumin was measured using a spectrum diagnostics colorimetric kit (catalog no. 210 001, Egypt). Then, the following equation is used to determine ionized calcium: Ionized calcium (mg/dl) = 0.9 + [(0.55 x total calcium (mg/dl)) – (0.3 x albumin (g/dl))] [15].
Furthermore, an enzyme linked immunosorbent assay (ELISA) kit purchased from Fortress Diagnostics was utilized to evaluate serum vitamin D_2 levels (catalog no. BXE0111A, UK).
Besides, fasting plasma glucose (FPG) level was assessed using a colorimetric kit purchased from spectrum diagnostics (catalog no. 250 001, Egypt).

2.2.2 DNA extraction and genotyping

The participants' genomic DNA was extracted from their entire blood using a DNA blood extraction kit (GeneJET™ Whole Blood Genomic DNA Purification Mini Kit, Thermo Scientific, USA). The concentration and purity of genomic DNA in each sample were ascertained using NanoDrop™ 2000 UV-Vis spectrophotometer, Thermo Scientific, Wilmington, Delaware, *USA*. The purified DNA should have $A_{260/280}$ ratio 1.8 to 2. Degradation was avoided as DNA is stable. However, excessive shearing, repeated freezing and thawing are also avoided. Moreover, contamination was avoided as any sample had ratio of the absorbance at 260 and 280 nm ($A_{260/280}$) out of the range between 1.8 to 2 was excluded because this DNA sample was contaminated with protein which absorbs light at 280 nm.

In addition, most of the purified DNA samples had concentration of 20 ng/µL which was the recommended DNA concentration per PCR well for TaqMan® SNP (rs731236) assay.
The VDR Taq1 SNP rs731236 with an accession number NG_008731.1:g.65058 was assessed by allelic discrimination assay using real-time polymerase chain reaction (PCR). The Taq1 rs731236 TaqMan® probes were pre-designed with context sequence [VIC/FAM]: TGGACAGGCGGTCCTGGATGGCCTC[A/G]AT CAGCGCGGCGTCCTGCACCCCAG (nucleotide position: 65083-65033) by Applied Biosystems ID: rs731236, C_2404008_10; Applied Biosystems, California, USA. The primer sequences were F: 5′-CAGAGCATGGACAGGGAGCAA-3′ (nucleotide position: 64765 - 64785), R: 5′-CACTTCGAGCACAAGGGGCGTTAG-3′ (nucleotide position: 65265-65242). The final reaction volume per PCR well was 20 µL which

consisted of: TaqMan® Universal Master Mix supplied by Thermofisher, 2x (10 µL), TaqMan® SNP Genotyping Assay, 40x (0.5 µL) and DNA template + DNase-free water (9.5 µL).

Thermal cycling conditions were initiated by AmpliTaq Gold® polymerase activation at 95 °C for 10 minutes, followed by 45 cycles (denaturation at 95 °C for 15 seconds then annealing/extension at 60 °C for 1 minute) and fluorescence was detected using ViiA7™ detector (Applied Biosystems, California, USA) and then analyzed with the manufacture's software. Negative control and duplicate samples were included to ensure the accuracy of results and all scatter plots were checked visually to verify the accuracy of the genotype records.

2.2.3 Bone mineral density (BMD)

All participants had a DEXA scan of BMD (g/cm^2) at the femoral neck (FN) and were categorized according to World Health Organization (WHO) guidelines. A healthy group is described as a T-score of +1 to 1. A T-score of 1 to 2.5 suggests osteopenia. A T-score of -2.5 or less implies the presence of osteoporosis [16].

2.2.4 Statistical analysis

GraphPad prism® version 6.01 (GraphPad Software, San Diego, CA, USA) and Microsoft Excel 2016 were used for analyzing the data. Values were represented as mean ± standard deviation (SD), median, interquartile range (IQR) and percentage. Data of the studied groups were subjected to D'Agostino and Pearson omnibus normality test. The statistical differences between two groups were detected by Mann-Whitney test. The parametric Pearson correlation test or non-parametric spearman test was applied to find a correlation between 2 variables and correlation coefficient (r) was calculated. Statistical significance was considered if P-value < 0.05. Differences in genotype and allele frequencies between groups were analyzed using fisher's exact test, and the odd ratios (OR) with 95% confidence intervals (CI) were calculated. P-Value < 0.05 was considered statistically significant.

3. RESULTS

3.1 The demographic characteristics and the status of osteoporosis

The demographic characteristics and the status of osteoporosis of the participants are represented in table (1) where age and T score are presented as mean ± SD and the other parameters are presented as median and IQR. BMD represented by FN T score showed 1.2 ± 0.3 and -2.7 ± 0.5 in control group and secondary T2DM osteoporotic fractures, respectively. Moreover, the results showed statistically significant elevation of FPG level in secondary T2DM osteoporotic fractures compared to the control group (P < 0.0001). It showed statistically significant low levels of serum albumin, total calcium and vitamin D_2 in secondary T2DM osteoporotic fractures compared to the control group (P < 0.0001).

Table 1: The demographic characteristics and the status of osteoporosis of the studied groups.

	Control	Secondary T2DM osteoporotic fractures	P value
Number	45	45	
Age (years)	37.8 ± 2.17	37.9 ± 2.27	> 0.05
FN T score (x S.D.)	1.2 ± 0.3	-2.7 ± 0.5	<0.0001*
FPG (mg/dl)	99.5 (24)	165 (61)	<0.0001*
Total calcium (mg/dl)	10 (2.12)	8 (3.51)	<0.0001*
Albumin (g/dl)	3.6 (0.61)	2.87 (0.47)	<0.0001*
Ionized calcium (mg/dl)	5.08 (1.31)	4.33 (1.73)	0.0008*
Vitamin D_2 (ng/ ml)	38 (8.9)	1.23 (0.728)	<0.0001*

Age and T score were represented as mean ± SD. All biochemical markers were demonstrated as median (IQR).
FN: femoral neck, FPG: fasting plasma glucose.
* P < 0.05 significant difference compared to the control group.

3.2 Correlation between serum vitamin D_2 and other biochemical data

Table (2) shows the correlation between serum vitamin D_2 and other studied biochemical markers. Negative correlation was found between serum vitamin D_2 and FPG while positive correlation was observed between serum vitamin D_2 and both of total and ionized calcium in secondary T2DM osteoporotic fractures. Albumin is positively correlated with serum vitamin D_2 in secondary T2DM osteoporotic fractures compared to the control group as well.

3.3 Distribution of VDR Taq1 rs731236 A > G genotypes and alleles in the studied groups:

Table (3) demonstrates the genotype and allelic distribution of VDR Taq1 rs731236 A (T) > G (t) SNP analysis. G (t) allele and GG (tt) genotype were significantly found in the control group versus secondary T2DM osteoporotic fractures with P value <0.05. AG (Tt) is statistically non-significant in

secondary T2DM osteoporotic fractures compared to the control group.

Table 2: Correlation between serum vitamin D_2 and other assessed biochemical data of the studied groups.

	Control		Secondary T2DM osteoporotic fractures	
	r	**P**	**r**	**P**
FPG (mg/dl)	0.0121	0.9	-0.177	0.24
Total calcium (mg/dl)	-0.196	0.19	0.104	0.49
Albumin (g/dl)	-0.124	0.4	0.07	0.62
Ionized calcium (mg/dl)	0.156	0.3	0.171	0.26

* statistically significant at p< 0.05 using Spearman's correlation test.

FPG: fasting plasma glucose, r: correlation coefficient.

Table 3: Distribution of VDR TaqI rs731236 A>G genotypes and alleles in the studied groups

	Control	Secondary T2DM osteoporotic fractures	P value	OR (CI)
AA	13 (28.8%)	13 (28.8%)	1	reference
GG	19 (42.2%)	0 (0%)	0.002*	0.025 (0.0014-0.46)
AG	13 (28.8%)	32 (71.1%)	0.12	2.46(0.902-6.71)
AG+GG	32 (71.1%)	32 (71.1%)	1	1 (0.40-2.48)
A allele	39 (43.3%)	58 (64.4%)	1	reference
G allele	51 (56.6%)	32 (35.5%)	0.006*	0.42 (0.23-0.76)

OR: odds ratio; CI: confidence interval.

* statistically significant at p < 0.05 using fisher exact's test

3.4 Correlation between VDR Taq1 genotypes and the studied biochemical parameters

Table (4) expresses the correlation between VDR TaqI AA versus AG+GG genotypes regarding their biochemical markers. Negative correlation is observed between FPG and AG+GG genotypes compared to AA genotype in secondary T2DM osteoporotic fractures. Positive correlation is found between both total and ionized calcium and AG+GG genotypes compared to AA genotype in secondary T2DM osteoporotic fractures. Albumin is positively correlated with AG+GG genotypes compared to AA genotype in secondary T2DM osteoporotic fractures as well. Moreover, serum vitamin D_2 is positively correlated with AG+GG genotypes compared to AA genotype in secondary T2DM osteoporotic fractures.

Table 4: Correlation between VDR Taq1 genotypes and the studied biochemical parameters

	Control				Secondary T2DM osteoporotic fractures			
	AA	AG+GG	R	p	AA	AG+GG	r	p
FPG (mg/dl)	102 ± 11.8	93.3 ± 11.5	-0.55	0.04*	171 ± 32.7	165 ± 30.4	-0.092	0.71
Total calcium (mg/dl)	9.52 ± 1.04	10.1 ± 1.18	-0.27	0.33	8.28 ± 1.76	9.16 ±3.8	-0.175	0.51
Albumin (g/dl)	3.82 ± 0.44	3.69 ± 0.44	0.077	0.80	3.07 ± 0.323	3.01 ± 0.291	0.027	0.92
Ionized calcium (mg/dl)	4.84 ± 0.54	5.09 ± 0.783	-0.385	0.18	4.53 ± 0.91	5.03 ± 2.02	-0.194	0.46
Vitamin D_2 (ng/ml)	35.5 ± 5.72	41.7 ± 12.6	-0.146	0.62	0.986 ± 0.348	1.55 ± 1.73	0.270	0.37

* statistically significant at p< 0.05 using Pearson's correlation test.

FPG: fasting plasma glucose, r: correlation coefficient.

4. DISCUSSION

Vitamin D has pleiotropic functions through VDR binding. Not only does it maintain bone and calcium homeostasis but also, it has a critical role in insulin secretion. Therefore, vitamin D was noticed to be involved in T2DM pathogenesis and osteoporosis as well [10–12]. The VDR gene is found on chromosome 12q13.11, and has different SNPs including TaqI SNP on exon 9 [17].

A previous Egyptian study concluded that TaqI rs731236 has no influence on the risk of T2DM [14]. Moreover, premenopausal osteoporosis and osteoporotic fractures are rare and it occurs often as a secondary disease [18,19]. Accordingly, we aimed to study the influence of VDR TaqI rs731236 A>G on osteoporotic fracture as T2DM complication in Egyptian premenopausal adult females who are matched in their age and socioeconomic status.

Interestingly, the results showed the protective effect of the recessive genotype GG against secondary T2DM osteoporotic fractures where females carrying the recessive allele showed higher levels of serum ionized calcium and vitamin D_2. In a previous Egyptian study, the dominant allele of VDR TaqI (A) allele was associated with osteoporosis together with low calcium level but without affecting vitamin D level [20]. Moreover, positive correlation between vitamin D_2 and AG+GG genotypes compared to AA genotype in secondary T2DM osteoporotic group. However, another Egyptian study showed that the recessive allele was associated with lower vitamin D in obese Egyptian women[21]. Other studies showed that dominant allele is associated with lower bone mass density in postmenopausal women [22,23].

Furthermore, normal ionized calcium is associated with low serum vitamin D_2 in secondary T2DM osteoporotic fractures. It can be explained as calcium may be controlled by estrogen while estrogen increases tubular calcium reabsorption [24]. Hypovitaminosis D was noticed in women in their reproductive years despite sun abundance in Saudi Arabian study [25,26]. Moreover, negative correlation is found between serum ionized calcium and AG+GG genotypes compared to AA genotype. On the contrary, Greek postmenopausal females found that lower calcium is associated with G allele [27], while an Indian study stated that there is no VDR genotype association with calcium absorption [28].

Compared to the control group, healthy women have normal total and ionized calcium associated with sufficient level of vitamin D_2. Positive correlation is found between serum ionized calcium and vitamin D_2 as well. Nevertheless, total calcium is negatively correlated with vitamin D in the control group. It may be justified as vitamin D regulate calcium homeostasis through maintenance of ionized calcium [29], however total calcium may be affected by the level of estrogen through different stages of the menstrual cycle as it may increase in the follicular phase and decrease in the luteal phase [30].

Moreover, hyperglycemia was obviously shown in secondary T2DM osteoporotic fractures with negative correlation with vitamin D_2. Furthermore, FPG showed negative correlation with VDR TaqI AG+GG compared to AA genotypes. This results came in agreement with a meta-analysis that observed VDR TaqI variants was linked to the onset of T2DM in dark pigmented Caucasian [31]. In addition, uncontrolled hyperglycemia may accelerate bone resorption and decrease osteoblast activity [32]. Normal level of FPG and serum vitamin D_2 were observed in the control group. However, non-significant positive correlation is found between FPG and vitamin D_2 in control group with more vitamin D_2 level in AG+GG genotypes which support the protective hypothesis. It may be explained as optimal vitamin D level is needed for normal FPG as it affects insulin production [33]. Moreover, FPG level may be affected by estrogen and progesterone levels as it may be lower in the premenstrual phase due to increase glucose consumption by cells or it may be higher in the luteal phase due to increased progesterone and decrease insulin sensitivity [34,35]. Additionally, hypoalbuminemia was significantly found in secondary T2DM osteoporotic fractures. It is positively correlated with serum vitamin D_2 in the same group in this study which came in accordance with a Pennsylvanian study as it proved that osteoporotic fractures are linked to hypoalbuminemia [36].

Regarding control group, normal levels of serum albumin and vitamin D_2 was observed, however non-significant negative correlation was found in this group. It may be explained as the level of albumin may decrease to its low normal range in the luteal phase of healthy women [37].

Consequently, hypovitaminosis D_2 associated with the dominant allele of VDR TaqI rs731236 in T2DM women make it is difficult for vitamin D to attain its active form resulting in osteoporosis.

5. CONCLUSIONS

In brief, this study investigated the VDR TaqI rs731236 A>G SNP on the complication of T2DM with osteoporotic fractures. The recessive genotype was found to be protective against osteoporotic fractures in T2DM women. The present study recommends vitamin D to be added as an additive therapy in the treatment of T2DM females to decrease the risk of osteoporotic fractures.

Limitation of the study

Small sample size is a limitation. Moreover, estrogen level is also recommended to be assessed in the future studies.

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Conflicts of Interest: The authors declare that they have no conflict of interest.

Ethical Statement: Patients were recruited from Al-Azhar University hospitals "Sayed Galal and Al-Hussein Hospitals"- Orthopedic Surgery Department following the Helsinki declaration recommendations and the study was approved by the Review Board of the hospitals as well as the ethics committee (198) of the Faculty of Pharmacy (Girls), Al-Azhar University in Egypt.

Author Contribution: All authors have contributed in writing and editing the manuscript. NMA was responsible for investigation, methodology, resources, visualization, formal analysis and writing-original draft. AIA was in charge of conceptualization, data curation, investigation, methodology, project administration, supervision, validation, writing – review and editing. MIA was responsible for investigation, resources, validation, writing-review and edition, clinical assessment and recruitment of the study subjects. SSE was in charge of conceptualization, data curation, investigation, methodology, project administration, supervision, validation, writing - review and editing.

List of Abbreviations: T1DM: Type 1 diabetes mellitus; T2DM: Type 2 diabetes mellitus; BMD: Bone mineral density; VDR: Vitamin D receptor; DEXA: Dual energy X-ray absorptiometry; SNP: Single nucleotide polymorphism; PCR: Polymerase chain reaction; SD: Standard deviation; IQR: Interquartile range.

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