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Original article

Vitamin D status, receptor gene BsmI (A/G) polymorphism and breast cancer in a group of Egyptian females

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ABSTRACT

Background: Vitamin D is involved in a wide variety of biological processes including bone metabolism, modulation of the immune response, and regulation of cell proliferation and differentiation. The present study aimed to investigate vitamin D status and the genetic polymorphism BsmI (A/G) of vitamin D receptor (VDR) among a group of Egyptian female patients with breast cancer.

Methods: The current study included 60 female patients diagnosed as breast cancer (BC) attending Mansoura Oncology Center, Mansoura University, and 60 age-matched healthy control females. Serum 25(OH) vitamin D level was measured using Enzyme-linked immunosorbent assay (ELISA) kit. A polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) method and fragment analysis were performed to determine the VDR BsmI (A/G) polymorphism.

Results: 25(OH) vitamin D levels were significantly lower in the patients with BC (22.1 ± 10.9 ng/ml) compared to controls (41.2 ± 11.22 ng/ml) ($p \leqslant 0.001$). Vitamin D deficiency- insufficiency was reported in 76.7% of BC patients and 20% of the controls ($P \leqslant 0.001$, OR = 13.1, 95%CI = 5.5 – 31.4). Bb genotype was statistically higher in the BC patients than in the healthy controls ($P \leqslant 0.001$). 81.2% of BC patients were of Bb genotype, 10.9% of BB genotype and 4.3% of bb genotype, while in controls, 33.3% for each genotype. No statistically significant difference in allele frequency was observed between the two studied groups. Carriers of Bb genotype had 4.6 times increased risk of developing breast cancer (95% confidence interval of 2.0–10.3) when compared to other genotypes.

Conclusion: A significant association exists between vitamin D deficiency and the risk of breast cancer. B allele or Bb genotype of VDR may be a susceptibility risk factor for BC development.

1. Introduction

Vitamin D both from dietary and endogenous sources undergoes the first hydroxylation in the liver to form 25-hydroxyvitamin D [25(OH)D]. A second hydroxylation takes place in the kidneys and other tissues to form an active form of vitamin D (1,25-dihydroxyvitamin D) [1,25(OH)2 D] [1]. Vitamin D regulates calcium absorption in the small intestine and acts with parathyroid hormone to mediate bone mineralization and maintain calcium homeostasis in the blood. Studies have demonstrated a relationship between low vitamin D levels and various diseases; probably due to its anti-inflammatory and immune-modulating properties [2].

Interestingly, an active form of vitamin D (1,25-dihydroxyvitamin D) has been demonstrated to promote cell differentiation and inhibit cell proliferation, affecting cancer risk via binding to vitamin D receptor (VDR) [3]. VDR is expressed in many types of cells, including normal and malignant breast cells [4].

Breast cancer is strongly affected by the hormonal environment. Genetic variations responsive to hormonal activity are possible contributors for increased risk. The genes involved in steroid hormone metabolism and transport can act together to provide a high-risk profile for breast cancer. Variations in these genes can

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modify breast cancer risk via gene– gene or gene– environment interactions [5].

Breast cancer is the most common type of malignancy among females in Egypt and worldwide where it represents 38.8% of the total cancer incidence locally and 22.9% globally. In 2013, the estimated number of cases in Egypt was 17,905 and was expected to be triple by 2050 [6].

The autocrine/paracrine pathway of vitamin D biosynthesis has an important role in breast cell carcinogenesis [7]. In this pathway, circulating 25-OH vitamin D reaches the breast tissue to be converted into its active form by endogenous 1-α-hydroxylase that is present in the breast [7,8]. The locally produced active vitamin D binds to VDR and regulates cell development [8].

VDR is an intracellular hormone receptor, belonging to steroid hormone receptor family; it binds to 1,25(OH)2 D and interacts with specific nucleotide sequences (response elements) of target genes to produce many biological effects [9]. VDR gene lies on the long arm of chromosome 12 (12q12-14) and has approximately 200 single nucleotide polymorphisms (SNPs) [10].

Common allelic variants have been identified in human VDR gene with some of them having an essential risk for a variety of diseases including breast cancer [11]. The best-studied SNPs include a start codon polymorphism FokI (T/C) in exon II, BsmI (A/G) and ApaI (C/A) polymorphisms in the intron between exon VII and IX and a TaqI (T/C) variant in exon IX. These SNPs are strongly linked with a singlet (A) repeat in the 30-untranslated region of the gene that may influence VDR mRNA stability [12].

The present study has been conducted to compare vitamin D status as well as VDR BsmI (A/G) polymorphism in a group of Egyptian female patients with BC and age matched healthy control subjects.

2. Subjects and methods

2.1. Subjects

The present study included 120 participants: 60 female patients diagnosed as breast cancer admitted at Mansoura Oncology Center, Mansoura University,and 60 healthy age-matched females free of any benign or malignant breast diseases as a control group. Exclusion criteria were: chronic renal disease, liver disease, hyperthyroidism, malabsorption syndrome, patients taking drugs as anticonvulsants, glucocorticoids, immunosuppressant and intake of vitamin D within the last six months.

2.1.1. Diagnosis of breast cancer patients

Included patients were assessed by clinical examination, radiological examination by either ultrasonography or mammography for the breast mass in addition to adequate metastatic work up and the preoperative histopathological diagnosis was made via trucut biopsy or fine needle aspiration cytology. However, the study group preferred to rely on the tumor characteristics stated in the final postoperative histopathology records for the patients who were treated surgically. The study was approved by the Research Ethics Committee (REC) for the experimental and clinical studies at the Faculty of Medicine, Mansoura University, Mansoura, Egypt, Code number: R/16.03.116. The work has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans. All patients and healthy controls signed informed consent forms.

2.2. Sampling

A sample of five ml venous blood was withdrawn from the cancer patients and the healthy controls under complete aseptic conditions. One ml was added to EDTA containing tube for DNA extraction, and the remaining blood was added to a plain tube without anticoagulant, left for 10 minutes at room temperature to clot, and then serum was separated and stored at −20 °C until the time of assay of 25(OH) vit. D.

2.3. Anthropometrical measurements

Height and weight were measured, and then body mass index (BMI) was calculated as body weight divided by height squared (kg/m^2).

2.4. Measurement of serum levels of 25(OH) vitamin D

Serum 25(OH) vitamin D levels were measured using Enzyme-linked immunosorbent assay (ELISA) kit supplied by DRG, Division of DRG International, Inc. Fauenbergstr.18, D-35039Marburg, Germany.

Deficient/Insufficient subjects were defined as those having levels less than 30 ng/ml, while the sufficient ones were those with levels greater than30 ng/ml [13].

2.5. Determination of genetic polymorphism BsmI (A/G) of VDR

DNA was extracted from whole blood samples collected on EDTA using the Gene JET whole blood genomic DNA Purification Mini Kits (Thermo Scientific, lot 00138029, Lithuania, EU). The BsmI VDR polymorphism is located within intron 8 of the gene. It was determined using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis [14].

Genomic DNA from cases and controls was subjected to PCR analysis of VDR gene using the following primers for BsmI polymorphism; forward primer: 5′-CAACCAAGACTACAAGTACCGCGT CAGTGA-3′ and the reverse one: 5′-AACCAGCGGGAAGAGGT CAAGGG-3′.The reaction mixture contained 10 μl DNA templates, 3 μl of each primer, 25 μl master mix (Fermentas, Germany lot 00141171), 9 μl sterile high-quality water.

Reaction conditions were carried out in thermocycler PTC-100 (Biorad, USA) with the following cycling parameters: an initial 94 °C for 10 min followed by 35 cycle of 94°for 55 s (seconds) (denaturation), 66 °C for 70 s (annealing) and final extension at 72 °C for 10 minutes.

The resulting 800 base pair (bp) PCR product is then digested with BsmI at 65 °C for 18 h using 5 units of enzyme (Boehringer Mannheim, Penzberg, Germany) per 20 μl reaction. Following digestion, the DNA fragments were separated using 2% agarose gel containing ethidium bromide then, visualized under short-wave UV light and compared to those of DNA ladder run at the same time. DNA from homozygote individuals (BB) lacking a BsmI restriction site appeared on the gel as a single 800 bp band. DNA from homozygote individuals (bb) appeared as two well-separated bands, 650 and 150 bp, indicating that the BsmI enzyme site is present in both alleles. Heterozygotes (Bb) have three bands: a 650 bp band and a 150 bp band (representing the presence of the BsmI site in one allele) plus an 800 bp band (indicating its absence in the other).

2.6. Statistical analysis

The statistical analysis of data was conducted using the SPSS (Statistical package for social science) program (SPSS, Inc, Chicago, IL) version 20. Qualitative data were presented as number and percentage. Chi-square and Fisher's exact tests were used to compare groups. Quantitative data were presented by mean, SD (standard deviation). For comparison between two groups; student *t*-test was used. Deviations from Hardy–Weinberg equilibrium

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expectations were determined using the Chi-squared test. Odds ratio and 95% confidence interval were calculated. $P \leqslant 0.05$ was considered statistically significant.

3. Results

The study groups were matched regarding their age with an average age of (48.2 ± 13.2 years) for patients and (44.9 ± 11.22 years) for controls (P = 0.18).

Serum 25(OH)vit D level was significantly lower in the BC patients (22.1 ± 10.9 ng/ml) compared to healthy controls (41.2 ± 11.22 ng/ml) ($P \leqslant 0.001$) (Table 1), with 76.7% of the BC patients found to be (deficient/insufficient), while only 20% of the controls were (deficient/insufficient) with ($P \leqslant 0.001$, OR = 13.1, 95% CI = 5.5–31.4).

Except for BMI (FET, P = 0.04) and metastasis (FET, P = 0.009), there was no statistically significant correlation between all studied variables (Table 3) and the hypovitaminosis D status in BC patients.

There was no deviation from the expected Hardy-Weinberg equation for the BsmI genotypes in the control group (P = 0.6). Analysis of the distribution of VDR BsmI polymorphism demonstrated that, the heterozygous Bb genotype was statistically higher in the BC patients than in the healthy controls ($P \leqslant 0.001$). No statistically significant difference in allele frequency was observed between the two studied groups (P = 0.3) (Table 1).

Distribution of VDR BsmI genotypes in both cancer patients and controls who were deficient in vitamin D is illustrated in Table 2; in BC patients, 81.2% were of Bb genotype, 10.9% of BB genotype and 4.3% of bb genotype, while in controls, 33.3% for each genotype.

Analysis of the Odds ratio of the BsmI SNP of VDR revealed that, carriers of Bb genotype had 4.6 times increased risk of developing breast cancer with 95% confidence interval of (2.0–10.3) when compared to other genotypes (Table 2).

Table 2
Hypovitaminosis D according to VDR BsmI genotypes in the studied groups.

The parameter	Hypovitaminosis D N (%)	Normal vitamin D level N (%)	OR (95% CI)
BC			
Genotype			
Bb	39(81.2)	9(64.3)	3.1(0.7–12.0)
BB	5(10.9)	4(28.6)	0.3(0.1–1.3)
bb	2(4.3)	1(7.1)	0.5(0.05–7.0)
Allele			
b	43(46.7)	11(39.3)	1.36(0.6–3.2)
B	49(53.6)	17(60.7)	r(1)
Non-BC			
Genotype			
Bb	4(33.3)	24(50.0)	0.5(0.1–1.9)
BB	4(33.3)	11(22.9)	1.7(0.4–6.7)
bb	4(33.3)	13(27.1)	1.3(0.3–5.3)
Allele			
b	12(50)	46(56.9)	1.1(0.4–2.7)
B	12(50)	50(43.1)	r(1)

r = reference group, OR = odds ratio, CI = Confidence interval.
BC = Breast Cancer, Non-BC = Non Breast Cancer.

4. Discussion

The current study shows that among the studied groups of Egyptian female subjects, vitamin D deficiency was more common in patients with BC as compared to the age-matched control group, indicating a strong association between breast cancer risk and serum levels of vitamin D. Although 1,25-dihydroxyvitamin D is the active form, it is widely accepted that the measurement of circulating 25(OH) D provides better information on patients vitamin D status and allows its use in the diagnosis of hypovitaminosis [12].

These results are supportive of the association between vitamin D and the risk of breast cancer. The results of the current study are consistent with those reported by Imtiaz et al. [15] who showed that vitamin D deficiency was observed in 95.6% of Indian patients with breast cancer versus 77% in the control group (**P** < 0.001). Low circulating levels of 25(OH) vitamin D is hypothesized to decrease the local production of 1,25(OH) 2 D within the breast tissue increasing the risk of developing BC [16]. This can be explained by that, low circulating levels of 25(OH) D decreases the availability of the substrate for 1α-hydroxylase expressed within the breast tissue and responsible for autocrine/paracrine synthesis of 1,25 $(OH)_2D_3$ [16].

Lowe et al. observed that low levels of circulating 25(OH) D, either alone or in combination with BsmI VDR genotype, may increase the risk of breast cancer in a UK Caucasian population [17]. Conversely, Bertone-Johnson et al. stated that such association is insignificant [18].

Studies have also shown that women with serum levels of 25 (OH) vitamin D more than 50 ng/ml had a 50% lower risk of development of breast cancer as compared to those with serum levels less than 30 ng/ml [18–20].

Table 1
Vitamin D status, serum levels & VDR BsmI polymorphism of the studied groups.

The parameter	Patients with breast cancer N(%)	Healthy controls N(%)	Statistical test & significance	OR(95% CI)
Vitamin D status**				
Deficient/insufficient	46(76.7)#	12(20.0)	$\chi^2 = 38.6$,	13.1(5.5–31.4)
Sufficient	14(23.3)	48(80.0)	$P \leqslant 0.001$*	1 (r)
Vitamin D level				
(Mean ± SD) (ng/ml)	22.1 ± 10.9	41.2 ± 11.22	t = 9.5, $P \leqslant 0.001$*	
Genotype				
Bb	48(80)	28(46.7)	$\chi^2$16.6, $P \leqslant 0.001$*	4.6(2.0–10.3)
BB	9(15)	15(25)		0.5(0.2–1.3)
bb	3(5)	17(28.3)		0.13(0.04–0.48)
H.W. equation	$\chi^2 = 22.8$, $P \leqslant 0.001$	$\chi^2 = 03$, P = 0.6		
Allele				
b	54(45)	62(51.7)	$\chi^2$1.1, P = 0.3	0.7(0.5–1.3)
B	66(55)	58(48.3)		

H.W. equation = Hardy – Weinberg equation.
r = reference group, OR = odds ratio, CI = Confidence interval.
* *P* is significant if ⩽ 0.05.
** Cut-off point = 30 ng/ml[13].

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Table 3
Factors associated with hypovitaminosis D in BC patients other than BsmI genotype.

The parameter	Total	Hypovitaminosis D N(%)	Significance
Cancer stage			
1&2	39	29(74.4)	
3&4	21	17(81.0)	FET, P = 0.75
Type			
IDC	48	36(75.0)	
Others	12	10(83.3)	FET, P = 0.7
BMI			
Overweight	10	5(50.0)	
Obese	50	41(82.0)	FET, *P* = 0.04*
Marietal status			
Single	1	1⟨1 0 0⟩	
Married	59	45(76.3)	FET, P = 1.0
History of breast disease			
No	46	36(78.3)	FET, P = 0.7
Yes	14	10(71.4)	
History of breast cancer			
No	51	41(80.4)	FET, P = 0.2
Yes	9	5(55.6)	
Menopausal status			
Pre-menopausal	34	25(73.5)	χ^2 = 0.4, P = 0.6
Post-menopausal	26	21(80.8)	
Metastasis			
No	55	45(81.8)	
Yes	5	1(20.0)	FET, *P* = 0.009*
Skin complexion			
White	39	29(74.4)	
Black	21	17(81.0)	FET, P = 0.75
Dressing style			
Hijab	44	36(81.8)	
Niqab	16	10(62.5)	FET, P = 0.17
Milk intake			
No	37	27(73.0)	
Yes	23	19(82.6)	χ^2 = 0.7, P = 0.5
Daily sun exposure			
No	25	19(76.0)	
Yes	35	27(77.1)	χ^2 = 0.01, P = 0.9
OCP			
No	36	28(77.8)	
Yes	24	18(75.0)	χ^2 = 0.1, P = 0.8
HRT			
No	45	33(73.3)	
Yes	15	13(86.7)	χ^2 = 1.1, P = 0.5
Multivitamin intake			
No	54	40(74.1)	
Yes	6	6(100)	FET, P = 0.2

BMI: Body mass index, OCP: Oral contraceptive pills, HRT: Hormonal replacement therapy, FET: Fisher's exact test.
* *P* is significant if ⩽ 0.05.

The protective effects of vitamin D could be mediated via the estrogen pathway by down-regulation of the estrogen receptor (ER) decreasing the oestrogenic bioresponses such as cell growth [21]. Additionally, vitamin D has been linked to promoting cellular differentiation, decreasing tumor cell growth, stimulating apoptosis, and reducing angiogenesis [22].

The current study demonstrated that VDR genotype distributions were different in the two studied groups regarding BsmI SNP ($\chi^2$16.6, P ⩽ 0.001). Genotype Bb was over-expressed in 80% of patients with BC, in comparison to 46.7% in the healthy controls(OR: 4.6, 95%CI: 2–10.3); with decreased presentation of the other two genotypes in patients as compared to controls (15% for BB, 5% for bb in patients versus 25%, 28.3% in controls, respectively). Consequently, it could be hypothesized that Bb genotype is considered a risk factor for developing BC independent of vitamin D level and status as no significant relation observed between vitamin D levels and VDR BsmI genotypes.

Inspite of no statistical significant difference between the two studied groups as regard allele frequency ($\chi^2$1.1, P = 0.3) with [OR = 0.7, 95% CI (0.5–1.3)], patients with B allele carried a higher risk for BC development than the b allele.

The results of other studies investigating the association between VDR BsmI polymorphism and BC risk are inconsistent. Positive associations were reported by Bretherton-Watt et al. [23], and Guyet al. [24], in Caucasian subjects in the UK; showing that bb genotype was significantly over-represented in patients with BC. Similarly, a Japanese study identified that bb genotype was the most common genotype in the patient group with almost 4-fold increase in the risk of BC [25]. Furthermore, a recent meta-analysis suggested the same findings [26–30]. Two other studies reported a significantly increased risk for the BB carriers; one carried out on Hispanic population [31] and the other on Taiwanese population [32].Contrarily, a meta-analysis by Zhang and Song [9] showed no significant association between the BsmI polymorphism and BC risk.

These studies were carried out on different populations as African American, French Canadian and southwestern American populations [10,32–39]. The difference in ethnogenetic background between the Egyptian females and the other studied populations could partly explain the differing results.

The present study suggested that vitamin D level is a risk factor for BC development independent of different VDR BsmI genotypes.

Indeed, the present study reported an association between the hypovitaminosis D status in patients with BC and BMI (FET, P = 0.04) and metastasis (FET, P = 0.009). Previous studies showed that vitamin D deficiency is an independent risk factor for abdominal obesity in women [40]. Consistently, the expression levels of the 25-hydroxylase and the 1α-hydroxylase have been shown to be decreased by 71% and 49%, respectively, in the subcutaneous adipose tissue of obese subjects suggesting that the adipose tissue can metabolize vitamin D locally [41].

In conclusion, the current study shows a significant association between vitamin D deficiency and the risk of breast cancer among Egyptian females. We also report that B allele or Bb genotype may be susceptibility risk factors for BC development and that b allele may be protective independent of vitamin D levels. Larger population- based studies are warranted to confirm these results.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Study limitations

This is a single center study with small sample size due to cost limitations.

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