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Biochemistry Section

Vitamin D and Vitamin D Receptor FokI, ApaI, and BsmI Gene Polymorphisms and their Relation with the Risk of Breast Carcinoma: A Case-control Study

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ABSTRACT

Introduction: Breast cancer stands as the leading cause of mortality among women in developing nations. The potential role of Vitamin D in mitigating the incidence of breast cancer is thought to stem from its ability to impede cell proliferation by interacting with the Vitamin D Receptor (VDR). The VDR gene is responsible for encoding the VDR, which plays a pivotal role in mediating the effects of vitamin D.

Aim: To analyse vitamin D levels and the association of VDR FokI, ApaI, and BsmI genotypic distribution frequency with the risk of breast cancer.

Materials and Methods: The case-control study included 220 samples, including 110 breast cancer patients and 110 age-matched control women aged 30-70 years. The Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) genotyping was performed using Deoxyribonucleic acid (DNA) extracted from blood, and the circulating levels of 25-hydroxyvitamin D by case/control were estimated by chemiluminescence immunoassay.

Results: The 3' VDR polymorphism BsmI sequence showed minimal association with breast cancer risk. The bb genotype had a significantly lower odds ratio of 0.056 (p-value<0.05). Conversely, the BB and Bb genotypes exhibited no statistically significant associations with odds ratios of 1.76 (95% CI: 0.36-8.54; p-value>0.05) and 1.30 (95% CI: 0.27-6.25; p-value>0.05), respectively. Isolated analysis of the FokI variant revealed a significant association with increased breast cancer risk, with odds ratios of 5.49 (FF) and 6.00 (Ff), both demonstrating statistical significance (p-value<0.05), and a Chi-square value of 0.006. Additionally, the p-value for serum Vitamin D levels was found to be highly significant at p-value<0.001, indicating that the levels were significantly lower in individuals newly diagnosed with breast cancer compared to those in the healthy control group.

Conclusion: The study found a significant link between breast cancer susceptibility and VDR (FokI) polymorphism FF and Ff genotypes, with minimal impact observed for (BsmI) polymorphism bb genotype. This implies that certain genetic variations, especially in the FokI polymorphism of the VDR gene, are associated with an elevated risk of breast cancer.

Keywords: ApaI single nucleotide polymorphisms, Breast cancer, BsmI single nucleotide polymorphisms, FokI single nucleotide polymorphisms

INTRODUCTION

Every year, breast cancer leads the world in morbidity and mortality rates for women [1]. In India, this disease accounts for 30.1% of all female cancer cases (Globocan 2020; http://globocan.iarc.fr/). The Indian subcontinent has seen an increase in cancer incidence, mortality, and morbidity [2-5]. The increasing mortality is likely due to ineffective screening strategies, advanced-stage diagnosis, and inadequate medical facilities. Recent studies have indicated that vitamin D may play a role in the development of breast cancer. In several studies, there is evidence that low levels of 25-hydroxyvitamin D {25(OH)D} increases the risk of breast cancer and have also shown an association between dietary intakes, dietary supplements, and skin production of vitamin D and breast cancer risk [6-8]. Aside from the classical role of vitamin D in calcium and phosphorus homeostasis, calcitriol exerts anticancer properties through transcriptional and/or non genetic mechanisms [9]. The active vitamin D3 (1α,25-dihydroxy vitamin D3) exerts its biological effects via the VDR. It belongs to the family of nuclear receptors and is a ligand-dependent transcription factor [10]. Cell cycle arrest, senescence, differentiation, and apoptosis are induced by the Vitamin D-ligand VDR in a variety of tumour types [11].

There are several polymorphisms in the coding and non coding regions of VDR on 12q13.11. Numerous Single Nucleotide Polymorphisms (SNPs) have been identified in and around exons 2-9 as well as in the 3' UTR region of the VDR gene [12]. The most commonly studied SNPs are those containing Restriction Fragment Length polymorphisms (RFLPs) rs1544410, rs2228570, and rs7975232, determined by restriction endonucleases BsmI, FokI, and ApaI [13-15]. The VDR contains the BsmI SNP (A/G) in intron eight near the 3' end, and its effect on VDR protein expression and activity is unclear. However, in Caucasians, Chinese, and Japanese Americans, it is in strong linkage disequilibrium with a polyadenosine microsatellite repeat, which may affect mRNA stability or translation activity [16]. A FokI site present in the 5′ promoter region substitution results in thymine (T) to cytosine (C) that changes the first of two possible translation initiation sites, resulting in different-sized VDR proteins. An f allele is three amino acids longer than an F allele and transcriptionally less active [17]. An ApaI SNP (C/A) variable site is located in intron 8 of the VDR gene. VDR polymorphisms may alter expression and function in breast cells, thereby modulating breast cancer risk [18].

Several studies performed on Caucasian populations have given inconsistent results regarding FokI, BsmI, and ApaI SNPs and breast cancer risk [19-22]. There have been very few studies on Asian populations. An association was found between the FokI SNP and Japanese-American women in the Hawaii-Los Angeles

Multiethnic Cohort (MEC), but not in large Chinese studies or small Iranian studies [23-25]. A BsmI SNP was associated with Iranian women and Japanese-American women from the Multiethnic Cohort (MEC) study, but not with two Chinese studies [26]. The ApaI SNP showed mixed results in different populations and was primarily studied in African Americans, Caucasians, and Chinese [27-29]. In the present study, three polymorphisms, one from the 5` region (FokI) and two from the 3` region of the VDR gene (ApaI and BsmI), and vitamin D, were investigated to assess the association with the risk of breast cancer. The study aims to offer insights into genetic factors impacting breast cancer susceptibility for personalised risk assessment and prevention.

MATERIALS AND METHODS

The present study was a hospital-based age-matched case-control study conducted at the Cancer Research Institute, a tertiary care centre located at Jolly Grant, Dehradun, Uttarakhand, India during the period from the year 2020 to 2023. This study included 110 freshly diagnosed breast cancer patients and age-matched 110 healthy controls aged 30 to 70 years. The institute's ethics committee approved the study, which was conducted in accordance with all the provisions of the Declaration of Helsinki (Letter No. SRHU/HIMS/ETHICS/2020/193). A written informed consent was obtained from all study participants.

Inclusion and Exclusion criteria: The inclusion and exclusion criteria were primarily used to select patients. The inclusion criteria included breast cancer patients selected based on histopathological confirmation, both pre- and postmenopausal women, and excluding those on hormonal therapy, with other cancers, recent Vitamin D supplementation, or pregnant or lactating. Age-matched healthy female volunteers were included as controls.

Sample size estimation: The sample size was estimated using the n-Master software for a matched case-control study (1:1) matching. Assuming that the proportion of exposed controls is 50% and the level of significance is 5% with a power of 90% to detect a two-fold increase in risk. The minimum number of required discordant pairs is 110.

Data collection: A comprehensive proforma was used to capture demographics, co-morbidities, family history, and anthropometric information, as well as pertinent clinical information from our online hospital database. The tumour morphology was classified according to criteria; Elston and Ellis used architectural aspects, nuclear differentiation levels, and mitotic index based on the 8th edition of the TNM staging system for breast cancer developed by the American Joint Committee on Cancer (AJCC) [30]. The quality of genomic DNA was assessed through agarose gel electrophoresis, and VDR gene polymorphisms were genotyped using PCR-RFLP analysis.

The Vitamin D levels in serum were determined for all freshly diagnosed cases and controls using the Chemiluminescent Immunoassay (CIA) method by trained laboratory technicians. Serum 25(OH)D levels were classified based on our institution's laboratory reference standards as sufficient/normal (75-250 nmol/L), insufficient (50-<75 nmol/L), and deficient (<50 nmol/L) [31]. Results were expressed in nmol/L.

Genotyping analysis: FokI, BsmI, and ApaI genotyping utilised PCR-RFLP analysis, employing agarose gels for DNA quality confirmation. The FokI polymorphism was detected using the following primers:

Forward: 5'GAT GCC AGC TGG CCC TGG CAC TG 3' and Reverse: 5'ATG GAA ACA CCT TGC TTC TTC TCC CTC 3', yielding a 272 bp fragment spanning the FokI site (Raza S et al., 2019) [32]. The BsmI polymorphism was detected using the following primers: Forward: 5'CAACAAGACTACAAGTACCGC GTCAGTGA3' and Reverse: 5'AACCAGCGGGAAGAGGTCAAG GGG 3', generating an 825 bp fragment surrounding the BsmI site (Raza S et al., 2017) [33]. The ApaI-RFLP was detected by the following primers: Forward: 5′ CAG AGC ATG GAC AGG GAG CAA G 3′ and Reverse: 5′ CGG CAG CGG ATG TAC GTC TGC AG 3′, yielding a 352 bp fragment spanning the ApaI site (El-Shorbagy HM et al., 2017) [29]. The following conditions were used for the PCR: initial denaturation at 94°C for three minutes, followed by 34 cycles of cyclic denaturation at 94°C for one minute, annealing 50 seconds at 71°C for FokI, 71°C for ApaI, and 58°C for BsmI, then extension at 72°C for one minute and one final cycle of final extension at 72°C for eight minutes, and final hold at 4°C. After PCR, the amplified PCR products were digested according to the manufacturer's instructions with FokI, BsmI (New England Biolabs, USA), and ApaI (Promega). In 2% agarose, fragments were stained with ethidium bromide to determine whether the enzyme recognition site was present (lowercase) or absent (uppercase). The genotypes for VDR-FokI (FF, Ff, ff), VDR-BsmI (BB, Bb, bb), and VDR-ApaI (AA, Aa, aa) polymorphisms were assigned. Randomly selected samples of three genotypes including Homozygous dominant, recessive, and heterozygous were confirmed by SNP sequencing, and the results were 100% concordant.

STATISTICAL ANALYSIS

Statistical analysis, including odds ratios and Chi-square tests, were used to evaluate associations between specific VDR gene polymorphisms and breast carcinoma risk. Data entered into Microsoft Excel 2010 were analysed using statistical software version Statistical Package for Social Sciences (SPSS) 20.0. Normality was assessed by the Kolmogorov-Smirnov test. An Independent t-test was used for two groups, and Analysis of Variance (ANOVA) for more than two groups to compare mean differences. The deviation from Hardy-Weinberg Equilibrium (HWE) was tested for polymorphisms by examining the differences between genotype frequencies observed and those expected, utilising the χ^2 test. Descriptive statistics and graphical representations were used to enhance the result interpretation. It is considered statistically significant when the p-value <0.05, and statistically insignificant if the p-value >0.05.

RESULTS

In this hospital-based case-control study, 110 patients and 110 healthy controls were compared. Among the study participants, demographic characteristics and risk factors were analysed, with predominantly 37 (33.6%) cases and 40 (36.4%) controls falling within the 40-49 age range. Urban residency accounted for 70% of cases and 86.4% of controls, while rural and Semiurban areas had lower frequencies. Premenopausal status was balanced, with 58.2% of cases and 61.8% of controls. Normal BMI was observed in 58.2% of cases and a higher percentage in controls (85.5%). Notably, 12.7% of cases had a positive first-degree family history, contrasting with the absence of such history in controls [Table/Fig-1].

Based on TNM staging, tumour morphology among 110 breast cancer patients showed Grade-I tumours with one case having vitamin D <50 nmol/L, two cases with 50 to <75 nmol/L, and none with 75-250 nmol/L (p-value=0.433). For Grade-II tumours, 51 (67.1%) had <50 nmol/L, 20 (26.3%) had 50 to <75 nmol/L, and 5 (6.6%) had 75-250 nmol/L (p-value 0.433). Grade-III tumours had 21 (67.7%) with <50 nmol/L, 8 (25.8%) with 50 to <75 nmol/L, and 2 (6.5%) with 75-250 nmol/L (p-value 0.433). No significant associations between tumour grades and vitamin D levels were found, contributing to understanding the tumour grade-vitamin D status relationship in this patient population.

The genotypes and allele frequencies of FokI, ApaI, and BsmI were illustrated in [Table/Fig-2]. In both cases and controls, the HWE of

Characteristics		Cases (%)	Controls (%)
Age (years)	30-39	31 (28.2)	34 (30.9)
	40-49	37 (33.6)	40 (36.4)
	50-59	28 (25.5)	27 (24.5)
	60-69	13 (11.8)	9 (8.2)
	≥70	1 (0.9)	0
Areas	Rural	22 (20)	6 (5.5)
	Urban	77 (70)	95 (86.4)
	Semiurban	11 (10)	9 (8.2)
Menopausal	Premenopausal	64 (58.2)	68 (61.8)
	Postmenopausal	46 (41.8)	42 (38.2)
BMI Category	Normal	64 (58.2)	94 (85.5)
	Underweight	5 (4.5)	9 (8.2)
	Overweight	37 (33.6)	7 (6.4)
	Obese Class-I	4 (3.6)	0
First degree family history	Yes	14 (12.7)	0

[Table/Fig-1]: Demographic characteristics of breast cancer patients and their matched controls.

Ff and breast cancer risk. With a 95% confidence interval (CI), the odds ratios were 5.49 (1.72, 17.64) and 6.00 (1.83, 19.67) with p-values of 0.004 and 0.003.

The examination of the ApaI (rs 7975232) VDR polymorphisms revealed an amplification product with a size of 352bp [Table/Fig-4a]. On agarose gels, 352bp bands were genotyped as AA homozygotes. The homozygote (AA) produces 216bp and 135bp fragments, while heterozygotes (AA) display three fragments, 352bp, 216bp, and 135bp [Table/Fig-4b]. 40.9% were heterozygous Aa, 49.1% were homozygous AA, and 10% were homozygous aa with respect to the Apa1 polymorphism among 110 cases and 110 controls. The ApaI genotypes showed no association with breast cancer risk.

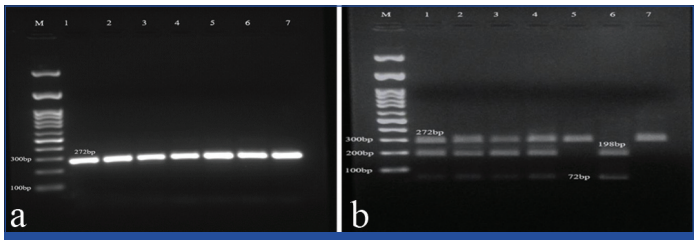
The analysis of BsmI (rs1544410) polymorphisms showed an amplified product size of 825bp [Table/Fig-5a]. Two or three fragments showing the Bsm1 restriction site (b) indicated intact amplification (B) reveals the absence. The undigested bands of 825 bp indicated a homozygous BB genotype. bb homozygotes produced two fragments (650bp and 175bp), and Bb heterozygotes produced three fragments (825bp, 650bp, and

Genotypes		Cases	Allele probabilities (p-value HWE)	Control	Allele probabilities (p-value HWE*)	OR (95% CI)	p-value*	χ^2 value
Genotypes (FokI)	FF	60	F=0.69, f=0.30 (0.15)	64	F=0.77, f=0.22 (0.15)	5.49 (1.72-17.64)	0.004	0.006
	Ff	32		42		6.00 (1.83-19.67)	0.003	
	ff	18		4		-	0.181	
Genotypes (ApaI)	AA	54	A=0.69, a=0.30 (0.15)	62	A=0.75, a=0.24 (0.15)	2.87 (0.92-8.97)	0.069	0.241
	Aa	45		43		2.31 (0.72-7.37)	0.157	
	aa	11		5		-	0.518	
Genotypes (BsmI)	BB	47	B=0.69, b=0.30 (0.15)	55	B=0.73, b=0.26 (0.15)	1.76 (0.36-8.54)	0.482	0.546
	Bb	59		52		1.30 (0.27-6.25)	0.743	
	bb	4		3		0.056	0.009	
Total		110		110				

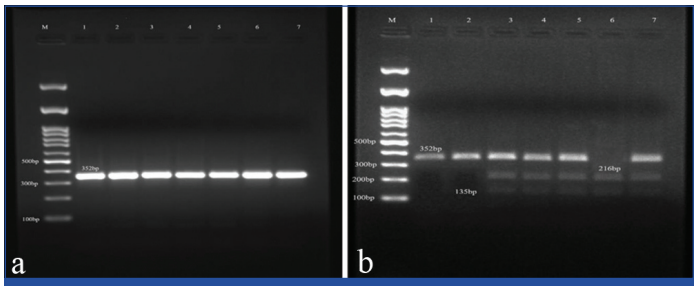
[Table/Fig-2]: An association of the genotypes of breast cancer patients and controls was made in the study.
*Hardy Weinberg equilibrium, *p<0.05, Significant

0.15 indicates equilibrium. In accordance with HWE, genotypic data were found to be reliable, indicating that selection or genetic drift has little influence on the genotypic distribution.

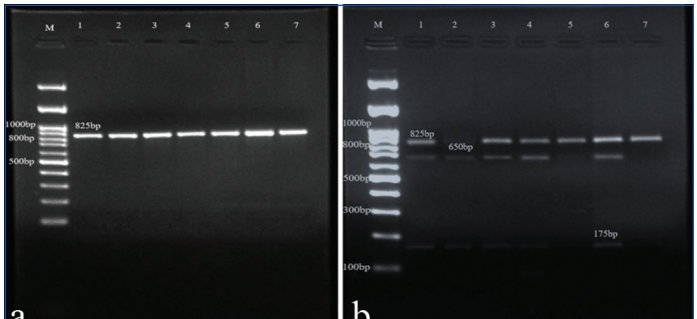
An analysis of FokI VDR polymorphisms yielded the amplification product with a size of 272bp [Table/Fig-3a]. An amplification product without FokI restriction site (F), while present in two or three fragments, indicates FokI restriction site (f). A non digested, single 272bp band genotype FF as homozygous, while Homozygotes (ff) showed two fragments of 198 and 72 bp, and heterozygotes (Ff) showed three fragments of 272, 198, and 72 bp [Table/Fig-3b]. The distribution of polymorphism in VDR FokI showed that 29.1% constituted the Heterozygous Ff, 54.5% were Homozygous FF, and 16.4% presented as homozygous ff cases, whereas the corresponding Control group genotype frequencies were 58.2%, 38.2%, and 3.6%, respectively. There was a significant association between the FokI genotypes FF and



[Table/Fig-3]: A 2% agarose gel stained with ethidium bromide depicts an amplification product of 272bp was obtained for Fok1. Ladder 100bp is Lane M. Lanes 1-7 show 272bp FokI amplified PCR products (a). F (T allele) is in upper band, C (C allele) is in lower bands. 100bps ladder on lane M. Lanes, 1-3, represent Ff heterozygotes, Lanes 4-6, represents FF homozygotes and Lane 7 represent ff homozygotes (b).



[Table/Fig-4]: A two percent agarose gel stained with ethidium bromide revealed an amplification product of size 352bp for ApaI polymorphism. Lane M shows a 100bp ladder and lanes 2-7 show a 352bp product (a); The upper band indicates A (C allele), lower band indicates a (A allele). 100bp ladder in lane M. There are Aa homozygotes in Lanes 1,2 and Aa heterozygotes in Lanes 3,4,5,7 and aa homoygote in Lane 6 (b).



[Table/Fig-5]: A two percent agarose gel stained with ethidium bromide revealed an amplification product of size 825bp for BsmI polymorphism. Lane M shows a 100bp ladder and lanes 2-7 show a 825bp product (a). The upper band indicates B (A allele), lower band indicates b (G allele). 100bp ladder in lane M. There are BB homozygotes in Lanes 5,7 and Bb heterozygotes in Lanes 1,3,4,6 and bb homoygote in Lane 2 (b).

175bp) on agarose gel [Table/Fig-5b]. Bsm1 polymorphism cases comprised 53.6% heterozygous Bb, 42.7% homozygous BB, and 3.7% homozygous bb. Additionally, 47.3%, 50%, and 2.7% of the control groups were genotyped.

A comparative analysis of vitamin D levels in breast cancer patients and the control group indicated mean values and standard deviations. Breast cancer patients had a mean vitamin D level of 43.54±19.58 nmol/L, while the control group had a higher mean of 89.89±26.13 nmol/L. The independent t-test showed a highly significant p-value of <0.001, signifying a substantial difference.

These findings highlight potential biomarkers for breast cancer, suggesting that low levels of vitamin D are related to increased breast cancer risk and lead to implications for diagnosis and prognosis in this patient population.

Moreover, a comparative analysis of genotypes and vitamin D levels in breast cancer patients and the control group, focusing on specific genotypes (FF, Ff, ff for FokI; AA, Aa, aa for ApaI; BB, Bb, bb for BsmI) was conducted. Mean vitamin D levels with standard deviations were assessed for both cases and controls. It shows significance in uncovering potential associations between distinct genetic variations and vitamin D levels. Significant differences in mean Vitamin D levels were observed among both cases (p-value=0.006) and controls (p-value=0.001) for different genotypes of BsmI. The data suggested varying degrees of association between genotypes and vitamin D levels [Table/Fig-6].

Genotypes		Cases	Control
		Mean±SD	
FokI	FF	42.98±21.32	46.33±26.08
	Ff	55.58±19.04	72.96±25.59
	ff	81.77±16.40	114.71±18.66
	p-value*	0.756	0.067
ApaI	AA	43.66±17.37	48.90±19.14
	Aa	65.39±21.56	67.90±27.14
	aa	89.54±19.43	102.67±21.42
	p-value*	0.316	0.001
BsmI	BB	44.76±22.41	87.70±24.24
	Bb	56.71±15.52	88.02±22.23
	bb	72.23±16.79	162.63±6.05
	p-value*	0.006	0.001

[Table/Fig-6]: Comparison of genotypes and Vitamin D levels (nmol/L) among Breast cancer patients and control groups.
*One-way ANOVA test, *p<0.05, significant

DISCUSSION

Breast cancer prevails as the predominant cancer in women globally and in India, with an age-adjusted prevalence of 25.8 cases per 100,000 women and a fatality rate of 12.7 per 100,000 women [34]. While developed regions still show higher occurrence rates, emerging countries, including India, face increased death rates from breast cancer [35]. Age is a significant risk factor, and present study indicates a notable prevalence of 33.6% in women aged 40-49. Tumour size and lymph node involvement are pivotal prognostic factors, with a common occurrence of lymph node infiltration at diagnosis (30-50% of cases). Metropolitan regions, age 50-59, and premenopausal status were prominent in these cases.

Present study revealed a significant association between the FokI genotype and the occurrence of breast cancer within the study population. Specifically, the FF genotype showed a substantial increase in breast cancer risk (OR: 5.49, p-value: 0.004), while the Ff genotype was associated with a significant risk reduction (OR: 6.00, p-value: 0.003). Conversely, the ff genotype was less frequent in the control group compared to the case group. These findings are consistent with Mishra DK et al., study on African American and Hispanic populations, as well as Chakraborty M et al., study within the Indian population, highlighting the elevated risk associated with Fok1 FF and Ff genotypes in breast cancer susceptibility [36,37]. In this study, analysis of the ApaI genotype distribution revealed a lower frequency of AA genotypes in cases (OR: 2.87, p-value: 0.069), with Aa genotypes showing a slightly higher frequency (OR: 2.31, p-value: 0.157). However, no significant association with breast cancer risk was observed for ApaI genotypes. This aligns with the ongoing study by Ahmed JH et al., on the African population, where no significant association was found between the Apa1 polymorphism and the condition under study [28].

Examining BsmI genotypes in present study, it was found that there were no significant associations with breast cancer risk. The ApaI genotypes (Aa and aa) revealed notable differences in vitamin D levels between cases and controls (p-values: 0.316 and 0.001), suggesting a potential relationship with breast cancer. These findings were in line with Reimers LL et al., population-based case-control study conducted on Long Island, New York, emphasising the influence of vitamin D-related gene polymorphisms on breast cancer susceptibility [38]. However, BsmI genotypes in present study showed no significant association with vitamin D levels. The p-value for serum vitamin D levels was highly significant at 0.001, signifying a substantial decrease in levels among individuals newly diagnosed with breast cancer compared to those in the healthy control group within our studied population. In a study by Ingles SA et al., African-American women with LS and LL poly(A) variations demonstrated a 50% lower risk of breast cancer than those with the SS genotype, particularly in the presence of the FF (FokI) mutation [39]. Whitfield KG et al., study on human fibroblast cell lines highlighted the statistical significance of VDR activity when both FokI and poly(A) genotypes were considered together [40]. Present study, aligning with previous research, conducted a comparative analysis of FokI, ApaI, and BsmI genotypes along with vitamin D levels (<50, 50 to <75, and 75 to 250 nmol/L) in breast cancer patients and controls [41,42]. Notably, statistical comparison between the groups for vitamin D levels for those carrying ApaI genotypes (Aa and aa) showed significant differences in vitamin D concentrations in cases and controls, and BsmI genotypes (Bb and bb) showed significant differences in vitamin D concentrations in cases and controls, suggesting that vitamin D status may be influenced by these genotypes.

Limitations

As with any research study, the present study has both strengths and limitations. For the studied analysis, only a few factors were taken into account, and quite a few factors were unmatched. Moreover, only a few VDR polymorphisms are considered for the 5' and 3' ends of this gene. Despite these limitations, this study significantly advanced the understanding of VDR polymorphisms (FokI, BsmI, and ApaI genotypes) and breast cancer risk.

CONCLUSION(S)

This study identifies an association between VDR (FokI) polymorphism FF and Ff genotypes and minimal impact for (BsmI) polymorphism bb genotype in breast cancer susceptibility. These findings could be useful in predicting breast cancer risk or whether a woman who has breast cancer will develop metastases. Highly significant serum vitamin D levels between breast cancer and control groups highlight the significant influence of VDR polymorphisms, particularly FokI, stressing the need for comprehensive studies across diverse ethnic populations to understand VDR gene variations' impact on breast cancer development thoroughly. Moreover, consideration of prognostic risk factors is needed for therapeutic applications

in the context of breast cancer. Vitamin D's potential preventive role in breast cancer, achievable through safe and affordable supplementation, emphasises its modifiability. The documented link between vitamin D deficiency and increased breast cancer risk underscores its public health significance, necessitating larger-scale investigations. VDR abundance in breast cancer tissues suggests potential treatment targets. Research on VDR FokI polymorphism gains importance, considering its potential moderation by family history.

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