Original Article

Association of Carcinoma Breast with Vitamin D serum Levels and Polymorphism in Vitamin D receptor Gene Apa1

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Abstract

Background: Breast cancer is the most common malignancy among women worldwide, and its incidence is increasing in South Asian populations, including Pakistan. While environmental and reproductive factors are well-recognized contributors, genetic susceptibility also plays a vital role. The vitamin D receptor (VDR) gene, particularly its ApaI single nucleotide polymorphism (SNP), has recently been explored for its possible involvement in cancer risk through altered vitamin D signaling.

Objective: To check the association between breast cancer, serum vitamin D levels, and polymorphism in the ApaI site of the VDR gene among Pakistani women.

Methodology: This cross-sectional comparative study included 115 healthy controls and 95 histologically confirmed breast cancer patients. Serum 25(OH) vitamin D levels were measured, and VDR ApaI genotyping was carried out using Polymerase Chain Reaction–Restriction Fragment Length Polymorphism (PCR–RFLP) analysis. Data were entered and analyzed using IBM Statistical Package for the Social Sciences (SPSS) version 21.

Results: The mean serum vitamin D level in all study subjects was 25.65 ± 13.45 ng/ml. Breast cancer patients had significantly lower levels (22.16 ± 7.95 ng/ml) than controls (29.82 ± 17.1 ng/ml). The "Aa" and "aa" genotypes of the ApaI polymorphism were more frequent among cases. In the dominant model, carriers of Aa or aa genotypes had about a twofold higher risk of breast cancer (adjusted OR = 2.03, 95% CI: 1.07–3.86; p = 0.02). Moreover, breast cancer patients with the "Aa" genotype showed markedly lower vitamin D levels ($p < 0.001$).

Conclusion: Low serum vitamin D levels and the ApaI polymorphism in the vitamin D receptor (VDR) gene are significantly associated with breast cancer in Pakistani women.

Keywords: Vitamin D Receptor, Gene Polymorphism, Breast Cancer, Single Nucleotide Polymorphism,

Introduction

Breast cancer (BC) remains the most frequently diagnosed malignancy and a leading cause of cancer-related death among women globally. According to GLOBOCAN 2020, approximately 2.3 million new cases and over 685,000 deaths were reported worldwide, with a growing burden in low- and middle-income countries (LMICs).[1] Although once considered a disease of the developed world, BC incidence is now rising in South Asia, particularly in Pakistan, where it constitutes the most common cancer among females and accounts for nearly one in nine women being at risk during their

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lifetime.[2] Among Asian countries, Pakistan shows one of the highest age-adjusted rates of breast cancer.[3] BC is a multifactorial disease influenced by environmental, reproductive, hormonal, and genetic factors.[4] One emerging biological determinant is vitamin D (25-hydroxyvitamin D, or 25(OH)D), a fat-soluble hormone. It is known to regulate calcium-phosphorus homeostasis and exhibit immunomodulatory, anti-proliferative, anti-angiogenic, and pro-differentiation effects in various tissues, including breast epithelium.[5] Deficiency of vitamin D has been linked not only to chronic disorders such as diabetes, cardiovascular, and autoimmune diseases, but also to increased risk and poor prognosis of several malignancies, including breast, prostate, and colorectal cancers.[6]

The biological effects of vitamin D happen mostly through its nuclear receptor, the vitamin D receptor (VDR), which works as a ligand-activated transcription factor. When active vitamin D ($1,25(OH)_2D$) binds to VDR, it changes shape and controls the expression of genes involved in cell growth, differentiation, and apoptosis.[5] Studies have found that VDR expression is lower in breast tumor cells compared to the nearby normal tissue, which suggests that VDR may have a protective role against cancer.[7] Polymorphisms in the VDR gene may alter receptor function, transcriptional activity, or mRNA stability, thereby modulating vitamin D signaling and influencing cancer susceptibility. Among these, ApaI (rs7975232), located in intron,[8] is one of the most widely studied VDR variants. ApaI does not alter the amino acid sequence but may affect mRNA processing and stability.[8] Multiple studies have evaluated the association between VDR gene polymorphisms, including ApaI, FokI, BsmI, and TaqI and breast cancer risk, but results remain inconsistent and often population specific.[9]

Pakistan is a bit of a paradox because even with plenty of sunlight, vitamin D deficiency is very common, maybe due to clothing habits, staying indoors, and not enough fortified foods. Recently it has been reported that about 80–90% of adults, including women, are deficient in vitamin D.[10] So far, many studies have assessed the associations between breast cancer risk and polymorphism in VDR gene with inconsistent results in genetically different populations. Some shows positive association while others show negative association.[11,12] Most of these studies have been done in Caucasian populations. Very few have been carried out in South Asian populations. The interplay between VDR gene polymorphisms and hypovitaminosis D in breast carcinogenesis has been underexplored in South Asian populations. Therefore, the present study aims to check the association between breast cancer, serum vitamin D levels, and polymorphism in the ApaI site of the VDR gene among Pakistani women.

Methodology

The study was carried out at the Research Center in Endocrinology and Reproductive Sciences, Department of Physiology & Cell Biology, University of Health Sciences, Lahore. It was designed as a cross-sectional comparative study, conducted over a period of twelve months following approval from the Ethical Review Board.

Ethical Consideration

Ethical approval for the study was obtained from the Ethical Review Committee of the University of Health Sciences, Lahore (No. UHS/Education/126-14-847, Approval date: 24/03/2014). Written informed consent was obtained from all participants prior to inclusion, and confidentiality of all personal information was strictly maintained throughout the study.

Keeping in view an earlier study by Dalessandri et al., 2012, the sample size was calculated based on the observed difference in VDR ApaI genotype frequencies and specified to achieve 90% statistical power with a two-sided $\alpha = 0.05$.[13] To improve precision and allow for potential sample loss, a total of 210 participants were targeted. Ultimately, 95 histologically confirmed breast cancer patients and 115 age-matched healthy controls were recruited. The patients were enrolled from the surgical and oncology departments of tertiary care hospitals in Lahore, including the INMOL Cancer Hospital, while controls were selected from the general population.

A structured questionnaire was administered to all participants to record their demographic characteristics and known risk factors for breast cancer, including maternal age, parity and age at first childbirth, history and duration of breastfeeding, age at menarche and menopause (if applicable), and family history of breast cancer.

Inclusion Criteria

The study included newly diagnosed, histopathologically confirmed female patients of primary breast cancer, regardless of disease stage. Controls were age-matched healthy women, relatives of the recruited cancer patients, with no history of malignancy or metabolic syndrome.

Exclusion Criteria

Patients with any other concurrent malignancy, or those who had received chemotherapy or radiotherapy, were excluded. Participants who had taken vitamin D or calcium supplements were also excluded.

Following written informed consent, a detailed history and general physical examination were performed for each participant. Using a convenient sampling technique, 5 mL of venous blood was drawn from each subject, 3 mL was placed in EDTA-containing tubes and the remaining 2 mL in serum tubes. Estimation of serum 25-hydroxy vitamin D [25(OH)D_3] levels were measured using a total enzyme-linked immunosorbent assay (ELISA) kit (AESKULISA 25-OH-Vitamin D). A 96-well microtiter plate coated with monoclonal antibodies was used for the assay.

Whole blood of all the study participants was used for the isolation of DNA by adopting phenol/chloroform technique. For the purpose of genotyping, latest procedure of Polymerase chain reaction of Restriction Fragment Length Polymorphism (PCR-RFLP) was performed. Forward as well as reverse primers which were specific for Apa1 allele were used as shown in Table I.[14]

A final reaction mixture of 15uL of PCR was formed by adding 8µL of PCR master mix, 0.5µL of each forward and reverse primer, 4µL of nuclease free water and 2µL of 50ng template DNA. The enhancement of VDR gene fragments for Apa1 polymorphism was performed by denaturing them at 95ºC for 5 minutes. Then incubation of the samples was performed for 35 cycles of denaturing, annealing and chain extension. At 72ºC, the final step of elongation was performed for 5 minutes. The annealing temperature for Apa1 was 69 ºC as shown in Figure I.

Analysis of the final PCR products was performed by a mixture of ethidium bromide and 1.5% agarose gel and finally observing them with the help of a UV transilluminator. The product obtained was of 740bp for Apa1. Restriction digestion was performed by Apa1 Restriction enzyme (Thermo Scientific) at 37℃ for 16 hours. The digested product was loaded on 2.5% agarose for 60 min at 100 volts (Figure II). Results were visualized under UV transilluminator gel

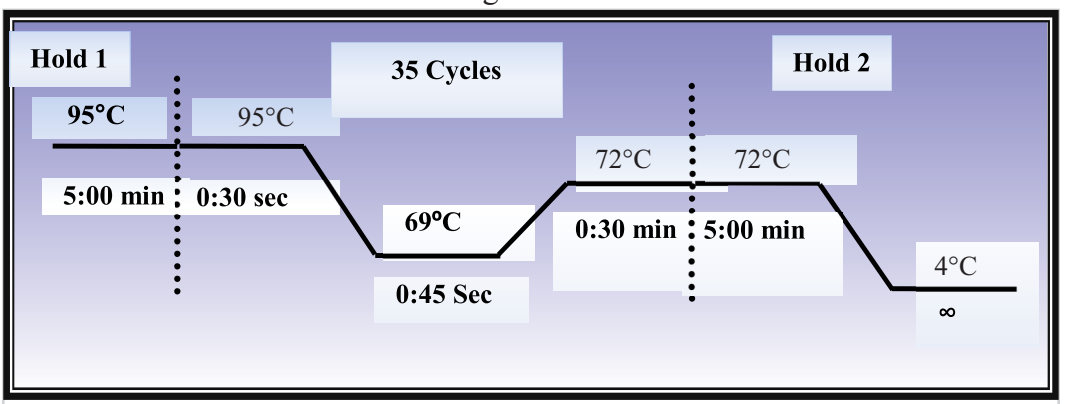

Figure I: *PCR cycle for Apa1 polymorphism*

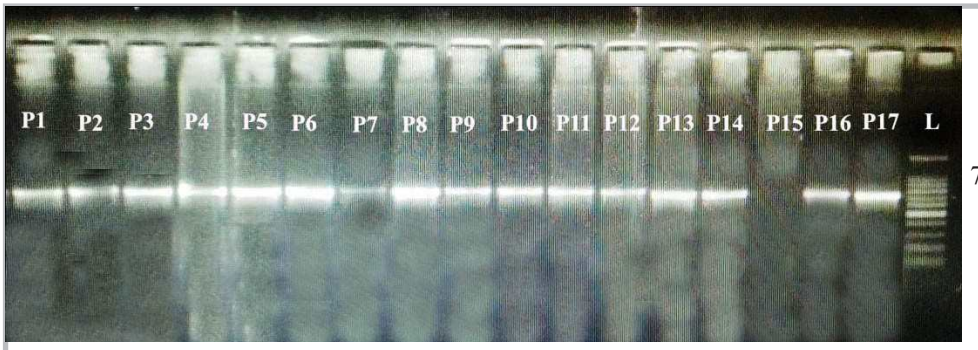

Figure II: *Ethidium bromide stained agarose gel for Apa1 PCR of VDR gene. Lane L shows 100 bp ladder. P stands for patient sample 1 -17.*

Table I: *Primer with GC content*

No	Primer	Sequence	GC content (%)	Annealing Temperature
1	*Apa1*-F	5´-AGA GCA TGG ACA GGG AGC AAG-3´	56%	69 ° C
2	*Apa1*-R	5´-GCA ACT CCT CAT GGC TGA GGT CTC-3´		

documentation system. The allele "A" was determined by the undigested product at 740bp whereas allele "a" was observed with digested product at 225bp and 515bp (Figure: III).

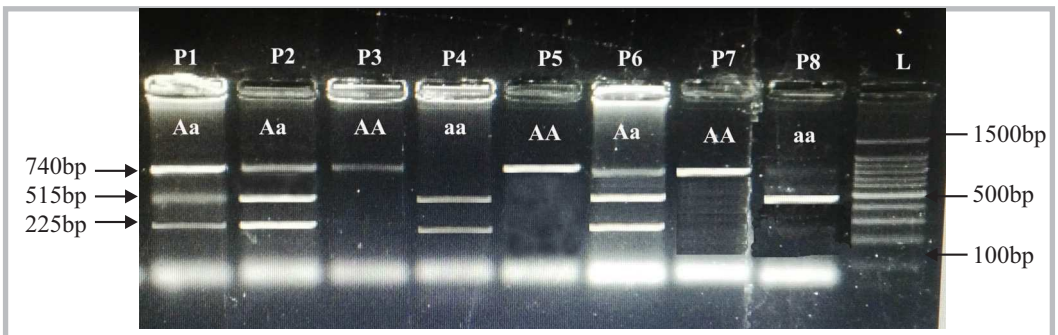

Figure III: *Ethidium bromide-stained agarose gel after RFLP for Apa1 polymorphism of VDR gene. All three possible genotype are visible. Lane L shows 1500bp ladder. P stands for patient samples 1-8*

Statistical Analysis

Data were entered and analyzed using IBM Statistical Package for the Social Sciences (SPSS) version 21. For the categorical data frequencies were taken while the numerical data was summarized in the form of Mean ± SD. Normality of the scale variables was checked by Shapiro-wilk test and depending upon the normality of the variables, parametric or non-parametric analysis was performed. For the comparison of the difference of mean for numerical parameters Independent Student's T-test was applied. The genotype distribution and evaluation of controls for Hardy-Weinberg equilibrium was calculated by using online calculator,[15] which helps in the analysis of various genotypic distributions i.e. dominant, recessive, co-dominant or log-additive as well as the association of the risk of disease development with every Single Neucltise Polymorphism (SNP). Logistic regression was conducted for both the crude and adjusted analysis. Mann-Whitney U test was applied for com-parison of the non-normally distributed Vitamin D levels in genome of study participants. In this study, p value less than 0.05 was set for statistical significance.

Results

The results show that the mean age of controls was 39.4

Table II: *Comparison of age, menarche age, marital status and family history of Ca breast in study groups*

Parameter		Over All n (%)	Controls n (%)	Cases n (%)	χ^2 (df)	p-value*
Age (years)	21-30	31 (14.8)	22 (19.1)	09 (9.5)	7.174 (3)	0.06
	31-40	66 (31.4)	40 (34.8)	26 (27.4)		
	41-50	66 (31.4)	32 (27.8)	34 (35.8)		
	>50	47 (22.4)	21 (18.3)	26 (27.4)		
Age of Menarche (years)	<=12	85 (40.5)	49 (42.6)	36 (40.5)	0.480 (1)	0.49
	>12	125 (59.5)	66 (57.4)	59 (62.1)		
Marital Status	No	16 (7.6)	09 (7.8)	07 (7.4)	0.015 (1)	0.90
	Yes	194 (92.4)	106 (92.2)	88 (92.6)		
Family History	No	197 (93.8)	109 (94.8)	88 (92.6)	0.414 (1)	0.52
	Yes	13 (6.2)	06 (5.2)	07 (7.4)		

*Chi-square test was applied to compare categorical variables between groups. A *p-value < 0.05 was considered statistically significant.*

Table III: *Comparison of Serum Vitamin D (Mean + S.D) levels of study subjects according to student 't' test*

Parameter	Overall (n=210)	Controls (n=115)	Cases (n=95)	p-value
	Mean ± SD	Mean ± SD	Mean ± SD	
Vitamin D (ng/ml)	25.65± 13.45	29.82 ± 17.1	22. 16±7.95	0.001*

*Mean + SD (Standard deviation), p-value calculated using Independent Samples t-test, *p-value <0.05 was considered statistically significant.*

±9.1 years, while the mean age of breast cancer patients was 43.6 ± 8.8 years. Most study subjects (62.8%) were between 31-50 years old. Age at menarche, marital status, and family history of breast cancer were similar between the two groups as shown in Table II.

Serum vitamin D levels were significantly higher in controls (29.82 ±17.1 ng/ml) compared to breast cancer patients (22.16 ± 7.95 ng/ml). as shown in Table III.

Table IV exhibits the genotype modeling to assess the association of Apa1 genotype with the disease. The Apa1 genotype distribution showed a slight increase in the "Aa" and "aa" genotypes among breast cancer patients compared to controls. Adjusted analysis showed an odds ratio of 2.03 (p=0.02) for the association between the "Aa" and "aa" genotypes and breast cancer.

The "Aa" genotype of the Apa1 polymorphism was associated with lower vitamin D levels in breast cancer patients compared to controls as shown in the Table V.

Table IV: *Genotype models for disease (Breast Cancer) association of Apa1 genotype*

Genotypes/ Models		Controls n (%)	Cases n (%)	*p*-value	OR crude [95% CI]	*p*-Value adjusted*	OR Adjusted* [95% CI]
Co-dominant Model	AA	83 (72.2)	58 (61.0)	0.23	1.00	0.07	1.00
	Aa	23 (20.0)	26 (27.4)		1.62 (0.84-3.11)		1.83 (0.90-3.75)
	Aa	09 (7.8)	11 (11.6)		1.75 (0.68-4.49)		2.64 (0.93-7.53)
Dominant Model	AA	83 (72.2)	58 (61.0)	0.08	1.00	0.02	1.00
	Aa & aa	32 (27.8)	37 (39.0)		1.65 (0.93-2.96)		2.03 (1.07-3.86)
Recessive Model	AA & Aa	106 (92.2)	84 (88.4)	0.36	1.00	0.12	1.00
	Aa	09 (7.8)	11 (11.6)		1.54 (0.61-3.89)		2.23 (0.80-6.22)
Over-dominant	AA & aa	92 (80.0)	69 (72.6)	0.21	1.00	0.18	1.00
	Aa	23 (20.0)	26 (27.4)		1.51 (0.79-2.86)		1.61 (0.80-3.24)
Log-additive	---	---	---	0.1	1.41 (0.93-2.13)	0.02	1.69 (1.07-2.68)

p-values were calculated using binary logistic regression analysis under co-dominant, dominant, recessive, over-dominant, and log-additive genetic models. Adjusted p-values and odds ratios (ORs) were obtained after controlling for age, body mass index (BMI), marital status, age at menarche, parity, history and duration of breastfeeding, use of hormonal therapy, menopausal status, and consanguinity. A p-value of <0.05 was considered statistically significant.

Table V: *Comparison of Vitamin D levels among different genotypes of study subjects*

Parameter		Vitamin D levels			Mann-Whitney U	p-value
		Over all	Controls	Cases		
		Median (IQR)	Median (IQR)	Median (IQR)		
Apa1 genotype	AA	27.14 (16.8)	22.77 (23.9)	28.74 (56.9)	1198.5	0.164
	Aa	18.52 (17.4)	35.15 (31.9)	14.91 (7.1)	62.5	< 0.000*
	aa	18.86 (11.9)	20.12 (20.5)	18.86 (11.1)	33.0	0.98

*Data are presented as Median (Interquartile Range, IQR). p-values calculated using the Mann–Whitney U test for comparison of vitamin D levels between cases and controls within each Apa1 genotype. *p < 0.05 was considered statistically significant.*

Discussion

This study provides evidence of a significant association between reduced serum 25(OH) vitamin D levels and increased risk of breast cancer in Pakistani women. The mean serum vitamin D level was notably lower in breast cancer patients compared to healthy controls.

Additionally, a higher frequency of the "Aa" and "aa" genotypes of the Apa1 polymorphism in the vitamin D receptor (VDR) gene was observed among breast cancer patients, and these genotypes were associated with significantly lower serum vitamin D levels. These findings underscore a potential gene- environment interaction that may influence breast cancer susceptibility. Several studies have established a protective role of vitamin D in breast tissue, with its anti-proliferative, pro-differentiation, and immune-modulating effects mediated through VDR signaling.[5,9] Low serum 25(OH)D levels have been associated with increased risk and poor prognosis in breast cancer, particularly in postmeno-pausal women.[16] The findings of the current study align with a large meta-analysis that demonstrated a dose–response inverse relationship between vitamin D status and breast cancer risk.[17] In the present study, 36.5% of cases were vitamin D deficient (<20 ng/mL) compared to 22.5% of controls, supporting these observations.

Findings from regional studies further reinforce this association. Ismail et al. reported significantly lower vitamin D levels in Egyptian women with breast cancer, where deficiency correlated with advanced tumor stage and nodal involvement.[18] Similarly, El-Shorbagy et al. also reported a negative prognostic impact of low vitamin D in Egyptian breast cancer patients.[19] Beyond vitamin D levels alone, genetic polymorphisms in the VDR gene may influence how vitamin D functions in the body. The ApaI polymorphism (rs7975232), located in intron 8 of the VDR gene, may affect mRNA stability and expression of the receptor.[20] In the current study, the dominant genotype model (Aa + aa) was significantly associated with increased breast cancer risk (adjusted OR: 2.03, $p = 0.02$). This is in agreement with Curran et al., who first reported an increased frequency of "Aa" and "aa" genotypes among breast cancer cases.[21] Yao et al. further demonstrated that combined effects of VDR polymorphisms and vitamin D deficiency may synergistically increase breast cancer risk, highlighting the importance of gene–environment interactions.[22] In this study, the "Aa" genotype was not only more prevalent in cases but was also associated with significantly lower serum vitamin D levels among patients. This suggests that the ApaI polymorphism may affect VDR function, potentially reducing vitamin D activity and contributing to the lower vitamin D levels observed in these individuals. Similar findings have been observed by Colagar et al., who reported lower vitamin D levels among women with "a" alleles of the VDR gene, further supporting the idea of a functional consequence of this SNP.[23]

The widespread vitamin D deficiency in Pakistan has been well documented, despite abundant sunlight. Cultural clothing practices, limited outdoor exposure, lack of food fortification, and insufficient dietary intake are likely contributing factors.[24] Given the high prevalence of vitamin D deficiency in the Pakistani population, this association becomes particularly significant, as recent research has demonstrated a strong link between lower vitamin D levels and an increased risk of breast cancer.[25] This deficiency may be especially concerning in genetically susceptible individuals, as suggested by our findings. VDR polymorphisms such as ApaI may also have implications for prognosis and therapeutic response. Therefore, screening for vitamin D levels and VDR genotypes could aid in risk stratification and guide potential preventive or therapeutic interventions.

Conclusion

In conclusion, this study demonstrates that both low serum vitamin D levels and the ApaI polymorphism in the vitamin D receptor (VDR) gene are significantly associated with breast cancer in Pakistani women. While vitamin D deficiency is well-recognized in the Pakistani population, our findings find the association of these low levels with breast cancer. These results emphasize the importance of exploring gene– environment interactions in disease prevention and suggest that maintaining adequate vitamin D status may have implications for breast cancer risk reduction.

Limitations and Recommendations

This study had certain limitations. The use of a convenience sampling technique may have introduced selection bias, thereby limiting the generalizability of the results to the wider population. Additionally, the cross-sectional study design restricts the ability to infer causality between serum vitamin D levels, VDR gene polymorphisms, and breast cancer risk. As serum vitamin D concentration was measured only once, potential fluctuations due to seasonal variation could not be accounted for. Moreover, the absence of tumor subtype information

(ER, PR, HER2) may have influenced the observed associations. The study also did not include other important vitamin D receptor polymorphisms such as FokI, BsmI, and TaqI, which might further elucidate gene – environment interactions relevant to breast cancer susceptibility.

Conflict of interest: None

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Authors Contribution

All authors have read the final version and are responsible for the integrity of the study.

SD: Conception of the idea, literature search, data analysis, writeup and revision.

SN & IA: Literature search, data interpretation and write up and review.

AI, RA & MAS: Data collection and analysis, write up and revision.