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## VITAMIN D RECEPTOR GENE *BsmI* POLYMORPHISM AND RISK OF BREAST CANCER

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**ABSTRACT :** Breast cancer is a leading cause of death among women, around the world. It is a multifactorial disease involving genetic and environmental factors. Vitamin D is known to modulate biological processes like immune response, bone metabolism, cell growth regulation. The protective actions of vitamin D in cancer development are only sparsely understood, however, evidence shows that vitamin D participates in cell growth regulation, apoptosis and cell differentiation. It has also been implicated in the suppression of cancer cell invasion, angiogenesis and metastasis. The cellular effects of Vitamin D are mediated via Vitamin D receptor (VDR), which is a member of the nuclear receptor superfamily and a key mediator in the vitamin D pathway. VDR is expressed in the breast tissue, and the involvement of (VDR) polymorphisms in breast cancer etiology has long been a topic of interest. Several studies have suggested an association between VDR gene polymorphisms and risk of breast cancer. This study aims to investigate the distribution frequency and association of VDR *BsmI* polymorphisms in North Indian breast cancer patients. In this study, 125 breast cancer patients and 125 healthy individuals were enrolled. The prevalence of *BsmI* alleles and the genotype frequencies in patients with breast cancer was similar to that in the normal population. Our data indicated no significant differences between the patients and control subjects. No significant association was observed between VDR *BsmI* polymorphism and risk of breast cancer occurrence.

**Key words :** Vitamin D Receptor, breast cancer, polymorphism.

### INTRODUCTION

Breast cancer is the most common type of female cancer worldwide, and represents about 23% of all cancers in women (Ferlay *et al*, 2012). The annual rates of incidence and mortality of breast cancer are increasing, making it a global cause of concern. About 63,410 cases of female breast carcinoma in situ and 74,680 cases of melanoma in situ were expected to be diagnosed in the United States in 2017 (Rebecca *et al*, 2017). In India, the incidence of breast cancer has even surpassed cervical cancer, which was earlier believed to have the highest rates of incidence among Indian women. Breast cancer has now become a leading cause of cancer death among Indian women (Kaarthigeyan, 2012). A number of global studies have reported a significant hike in cancer-associated mortality and morbidity in the Indian subcontinent (Babu *et al*, 2013; Ali *et al*, 2011; Srinath Reddy *et al*, 2005 and Balasubramaniam *et al*, 2013). A 13.82% increase in the rate of mortality and 11.54% increase in the rate of cancer incidence have been seen in India, due to breast cancer during 2008–2012 (Ferlay

*et al*, 2012).

Environmental exposure to lower level of UV radiation is an essential factor for the synthesis of vitamin D, and may also be a risk factor for higher incidence of cancers of many types including breast cancer (Finkelmeier *et al*, 2014). The photochemical synthesis of vitamin D3 (cholecalciferol) occurs cutaneously, where pro-vitamin D3 (7-dehydrocholesterol) is converted to pre-vitamin D3 (cholecalciferol). Pre-vitamin D3 is further hydroxylated in the liver through CYP2R1 to form 25-hydroxy cholecalciferol, the major circulating form of vitamin D (Khokhar, 2012). The activation of 25-hydroxy cholecalciferol takes place in the kidney, where it is converted to 1,25 dihydroxycholecalciferol (calcitriol), the most active form of Vitamin D. The cellular functions of Vitamin D are mainly modulated via the nuclear Vitamin D Receptor (VDR) (Deeb *et al*, 2007). VDR has been reported to be involved in Estrogen related pathways, immunomodulation, Insulin-like growth factor signaling and known to affect gene expression in a ligand-dependent manner (Yang *et al*, 2017). VDR is also reported to be

involved in micro-RNA directed post-transcriptional mechanisms (Thomas Lisse *et al*, 2013). It is expressed in the epithelial, stromal and immune cells in the normal mammary gland (Zinser and Welsh, 2004). VDR behaves as a DNA binding transcriptional factor and forms heterodimers with the Retinoid X Receptor, recruiting other transcriptional co-activators that regulate target gene transcription, including genes like hp21 and hFOXO1, which are involved in cell proliferation, differentiation, and apoptosis (Anderson *et al*, 2011).

Genetic polymorphisms are reported to be one of the major causes of inter-individual difference in cancer incidence and single nucleotide polymorphisms have been reported to be the most common variants (Dong *et al*, 2008). A DNA sequence variation occurring when a single nucleotide — A, T, C or G — in the genome, differs between members of a biological species or between paired chromosomes in humans, is known as a Single Nucleotide Polymorphism. A variation occurring in at least 1% of the population is considered as SNP. SNP variations may affect human response to chemicals, drugs, vaccines, pathogens and development of diseases (Carlson, 2008). Genetic variations in VDR gene between individuals may appear as variations in the rate of photochemical synthesis of Vitamin D in skin, its hydroxylation in liver, subsequent activation in kidney, transport, metabolism and degradation, which may finally affect the levels of vitamin D in the body. Epidemiological studies suggest that Vitamin D Receptor polymorphisms may be linked to the biological functions of Vitamin D. Functional genetic polymorphisms that lead to an alteration in the regulation of gene expression, are predicted to have a significant influence on disease pathogenesis (Pastinen *et al*, 2006). The physiological activities of Vitamin D are mediated via VDR. The allelic variants of the human VDR gene may therefore be risk factors for a variety of diseases including breast cancer. Genetic polymorphisms of VDR may modulate the risk of breast cancer by altering its expression as well as function in breast cell. These genetic differences may act as biological markers and help predict an individual's response to drugs, susceptibility to toxins and risk of developing a particular disease. More than 60 polymorphisms have been identified in the VDR gene located in the promoter region, in and around exon 2-9 and in the 3' UTR region (Fang *et al*, 2005 and Uitterlinden *et al*, 2004).

Situated at the 3' end of the VDR gene, the genotypes assigned to the *Bsm1* (A/G) polymorphisms are BB, Bb, and bb. The *Bsm1* VDR polymorphism is an intronic variant. It does not affect the amount, structure or function of the VDR protein, however, it is believed to be strongly

linked with poly (A) microsatellite repeats in the 3'UTR and may influence VDR mRNA stability (Nam *et al*, 2003; Suzuki *et al*, 2003; Huang *et al*, 2004; Oakley-Girvan *et al*, 2004; Watt *et al*, 2001 and Guy *et al*, 2004). A large number of studies have reported an association between the VDR *Bsm1* genotype and increased risk of breast cancer (Guy *et al*, 2003; Trabert *et al*, 2007; Ruggiero *et al*, 1998; Guy *et al*, 2003 and McCullough, 2007). Guy *et al* reported a 1.8 fold increase in the risk of breast cancer associated with the VDR *Bsm1* bb genotype in Caucasian women (Guy *et al*, 2003).

Three other studies associated the BB genotype with increased risk of breast cancer. These studies referred to Caucasian women (Ingles *et al*, 2000), Taiwanese women (Buyru *et al*, 2003) and Latinas (McCullough *et al*, 2007). Some other studies by Chen *et al* (2005), Wang *et al* (2013) and Zhang (2014) also revealed no significant difference in the prevalence of the *Bsm1* polymorphism in breast cancer patients (Chen *et al*, 2005; Wang *et al*, 2013 and Zhang and Song, 2014). Therefore, the relationship between VDR polymorphisms and breast cancer still remains controversial. The purpose of this study was to investigate the association of the VDR gene *Bsm1* polymorphism with breast cancer in the North Indian population.

## MATERIALS AND METHODS

### Ethics statement

Ethical approval for the study was obtained from the Institutional Ethics Committee, King George Medical University, Lucknow (Ref. no. 78th ECM IIB-Ph.D./P2). All participants signed an informed written consent prior to providing blood samples.

### Study population

We investigated the prevalence of VDR *Bsm1* (*rs1544410*) A/G gene polymorphism in 125 breast cancer patients and 125 age matched healthy control subjects, who were in follow up at the Department of Radiotherapy, King George Medical University, Lucknow. Any patient who had been histologically diagnosed for Breast Cancer with no other concurrent chronic disease, had no infection with HIV, HBV or HCV, and aged between 18 and 70 years was selected for the study. Among women attending antenatal check-ups, and blood bank donors at King George Medical University, a series of 125 age-matched healthy females were selected as controls. The mean age of cases and control groups were 44.47yrs and 40.88yrs, respectively.

### Blood sample collection and DNA Extraction

Whole blood specimens were collected in tubes containing EDTA. The Salting out procedure (Miller *et*

al, 1988) was used for the extraction of DNA from blood. Informed Patient Consent was obtained from the subjects after clearing the queries related to study and the benefits expected were delivered to the participants. A peer reviewed well drafted, study proforma providing patient familial information, personal and clinical details was filled for each participant.

### Genotyping of *BsmI* (A>G rs1544410)

Genotyping was performed by Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism analysis (RFLP). The 825-bp DNA fragment encompassing the (intron 8) *BsmI* polymorphic site was amplified using the primer pair described by Morrison (Morrison *et al*, 1994). Table 1 depicts the sequence of the primer pair used for PCR amplification. The conditions for PCR amplification were as follows: Initial denaturation step of 10 min at 94°C followed by 30 cycles of 94°C for 30sec, annealing at 57°C for 30 sec, and extension at 72°C for 30sec. Final extension was allowed at 72°C for 5min.

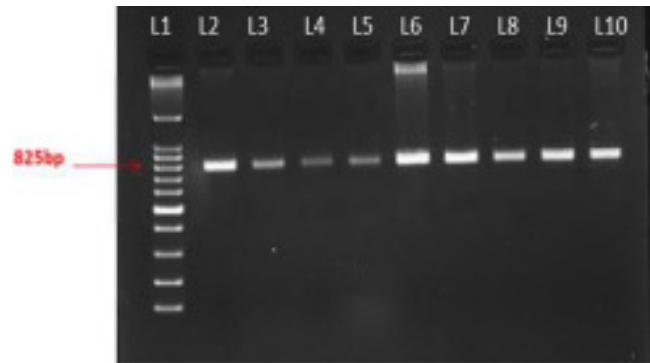
10µl of the 825bp amplification product was digested with 2.5U of *BsmI* restriction enzyme (New England Biolabs) and incubated at 37°C overnight. 6µl of the digested reaction mixture was electrophoresed for 1 hour at 100V and visualised on 2% agarose gels, stained with ethidium bromide. The size of the digested products was determined using a 100bp DNA ladder (G Biosciences). The presence of a given restriction site was assigned by lower case (b) and absence by upper case (B).

### Statistical method

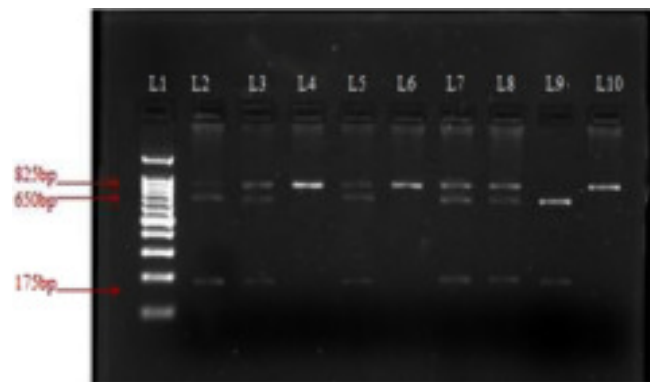
The difference in distribution of the genotypes or alleles between cases and controls was tested using the Chi-square statistic. Odds ratios (ORs) and 95% confidence intervals (95% CI) were calculated to evaluate the risk of breast cancer by comparing cases and controls. The association between the *BsmI* polymorphism and risk of breast cancer was evaluated using multivariate unconditional logistic regression analysis and p value <0.05 was considered as statistically significant. The statistical analysis was performed using the SPSS Software (SPSS Inc, version 17.0).

## RESULTS

PCR amplification yielded an 825bp product (Fig. 1). The 825bp PCR products were digested with *BsmI* restriction endonuclease (New England Biolabs) at 37°C



**Fig. 1 :** Ethidium bromide stained 2% agarose gel picture of *BsmI* PCR amplification products of VDR gene. The size of the amplification product for the *BsmI* polymorphism was 825bp. Lanes 2-10 show single bands corresponding to the 825bp PCR product; Lane 1 shows a 100bp ladder.



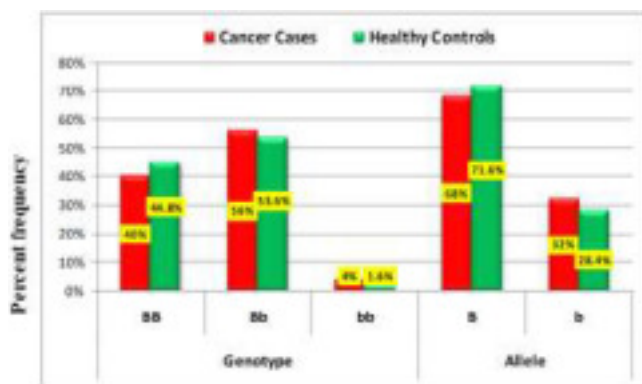
**Fig. 2 :** Ethidium bromide-stained 2% agarose gel picture of *BsmI* digested PCR products of VDR gene. The upper bands represent B (A allele), the lower bands represent b (G allele). Lanes 2, 3, 5, 7 and 8 show Ff heterozygotes; lanes 4, 6 and 10 show FF homozygotes; lane 9 shows ff homozygote; Lane 1 shows a 100bp ladder.

(Buyru *et al*, 2003), except that overnight digestion of the products using *BsmI* restriction endonuclease was allowed to minimise partial digests. Enzyme digestion was followed by electrophoresis in a 2% agarose gel (Fig. 2).

An intact amplification product indicated the absence of *BsmI* restriction site (B allele), while the presence of the *BsmI* site (b allele) was indicated by two or three fragments. The undigested, single 825bp bands were genotyped as BB genotype (homozygote of common allele) in the agarose gel. The bb genotype (homozygote of infrequent allele) generated two fragments of 650bp and 175bp while the heterozygotes (Bb) displayed three fragments (825bp, 650bp, and 175bp). Fig. 2 depicts the distribution of VDR *BsmI* polymorphism in cases and controls.

**Table 1 :** Primer sequences used for PCR amplification.

Primer	Sequence
<i>BsmI</i> Forward	5'-CAACAAGACTACAAGTACCGCGTCAGTGA-3'
<i>BsmI</i> Reverse	5'-AACCAGCGGGAAGAGGTCAAGGG-3'



**Fig. 3 :** The genotype, allele frequencies, in *BsmI* cases and controls.

**Table 2 :** Genotype and allele frequencies of VDR *BsmI* polymorphism in breast cancer cases and healthy controls.

<i>BsmI</i> A/G (rs1544410) SNP	Cancer Cases (n=125)		Healthy Controls (n=125)		OR	95% CI	$\chi^2$	p values	Bonferroni corrected p values	
	N	%	N	%						
Genotype	BB	50	40%	56	44.80%	0.821	0.50-1.36	0.59	0.443	1.000
	Bb	70	56%	67	53.60%	1.102	0.67-1.81	0.15	0.703	1.000
	bb	5	4%	2	1.60%	2.563	0.49-13.46	1.32	0.25	0.750
Allele	B	170	68%	179	71.60%	0.843	0.58-1.24	0.77	0.381	0.761
	b	80	32%	71	28.40%	1.186	0.81-1.74	0.77	0.381	0.761

Average Power = .816

The analysis included 125 breast cancer cases, and 125 healthy controls. The cases and controls having median age of 44.47yrs and 40.88yrs were selected from among the North Indian population. To evaluate the relationship between gene frequencies and genotype frequencies, Hardy-Weinberg equilibrium was tested. The distribution of VDR *BsmI* polymorphisms in patient and control groups is depicted in Table 2, showing genotype and allele frequencies of breast cancer cases and healthy controls. Three genotypes of VDR *BsmI* polymorphism were identified *i.e.* BB with 825 base pairs, bb with 650 and 175 base pairs, and the heterozygous Bb with 825, 650 and 175 base pairs as observed and categorized after enzyme digestion of the amplification products with the *BsmI* restriction endonuclease. It was observed that 56% of the patients were heterozygous (Bb) for the *BsmI* polymorphism, 40% were homozygous (BB) and 4% were homozygous (bb). The respective frequencies of these genotypes in the control group were 53.60%, 44.80% and 1.6% (Fig. 3). The results also identified B allele (68%) and b allele (32%) to possess the highest and lowest frequencies in cases, and the respective frequencies in controls were 71.60% and 28.40%. None of the *BsmI* polymorphisms in the Vitamin D Receptor gene were found to have a significant association ( $p > 0.05$ ) with the risk of breast cancer occurrence (Table 2). The differences in genotype frequencies of various

genotypes between cases and controls were tested to evaluate the association with breast cancer. The homozygous BB genotype associations between cases and controls were statistically insignificant ( $p=1.000$ ;  $\chi^2=0.59$ ; OR=0.821; 95% CI=0.50 -1.36). Similarly, for the Bb ( $p=1.00$ ;  $\chi^2=0.15$ ; OR=1.102; 95% CI=0.67 -1.81) and bb ( $p=0.75$ ;  $\chi^2=1.32$ ; OR=2.563; 95% CI=0.49 - 13.46) genotypes, no significant association with disease was observed.

## DISCUSSION

Association of the *BsmI* SNPs in the VDR gene with breast cancer risk was investigated in a data set from women participating in a study that included 125 breast cancer cases and 125 controls. For the homozygous (BB), the genotype frequency in cases was 40% however, the respective frequency in controls was 44.8%. These differences were not found to have statistical significance ( $p=1.000$ ;  $\chi^2=0.59$ ; OR=0.821; 95% CI=0.50 -1.36). The heterozygous (Bb) genotype had a frequency of 56% in cases and 53.60% in healthy controls. No statistical significance was observed for the Bb heterozygotes ( $p=1.00$ ;  $\chi^2=0.15$ ; OR=1.102; 95% CI=0.67 -1.81). A similar trend was observed for the homozygous bb genotype, the genotype frequency in cases and controls was 4% and 1.60% respectively. This data was also not found to be statistically significant ( $p=0.75$ ;  $\chi^2=1.32$ ; OR=2.563; 95% CI=0.49 -13.46). No overall association with breast cancer risk was observed for the VDR *BsmI* SNP. Till date, only a few studies with at least 100 cases and 100 controls have assessed associations of the VDR *BsmI* SNP with breast cancer risk in North Indian population. Breast cancer risk has strongly been related to endogenous hormone exposure and genes that are responsive to such hormones are possible candidates for being susceptibility genes. A number of studies have investigated candidate genes for breast cancer susceptibility. The VDR is a member of the steroid



hormone receptor superfamily and regulates gene transcription through interaction with hormone response elements in the promoter region of target genes. Ethnic variations are the genetic differences observed within and among populations because no two humans are genetically identical. Multiple variants of a given gene might exist in a given population. Ethnic variations are observed in the VDR polymorphisms (Uitterlinden *et al*, 2004 and Zmuda *et al*, 2000). A large number of studies have been conducted to investigate the relationship between VDR gene polymorphisms and risk of breast cancer occurrence. Various studies conducted to assess the association showed various outcomes and controversial results. A 1.5-fold increased risk of breast cancer for post-menopausal carriers of the bb genotype among Caucasians (OR=1.53) was reported by Trabert *et al*, (2007) but a similar association was not found among African-American women. A study by Ingles *et al* (2000) reported that the Bb and BB genotypes showed a 1.6-fold and 2.2-fold increased risk to breast cancer as compared to the bb genotype (OR=1.6, OR=2.2, respectively) (Ingles *et al*, 2000). The *Bsm1* polymorphism in intron 8 region of the VDR gene is known to alter the stability of VDR mRNA and subsequently, the levels of VDR protein (Sinotte *et al*, 2008). Some studies have identified the correlation of BB genotype (no enzyme restriction site) with cancer occurrence, while others reported the presence of the bb genotype as a factor increasing the risk for breast cancer. It has been reported that low levels of vitamin D in plasma and/or along with the *Bsm1* polymorphisms of the VDR gene may increase the risk of breast cancer incidence (Lowe *et al*, 2005). Our study identified no significant association between the *Bsm1* polymorphism and risk of breast cancer occurrence in the participating North Indian population. Some similar studies on Chinese and Pakistani populations did not observe any relationship between *Bsm1* VDR polymorphism and risk of breast cancer incidence (Rashid *et al*, 2015). However, a study on a Japanese-American population reported the association of VDR *Bsm1* polymorphism with the risk of breast cancer occurrence (McKay *et al*, 2009). Similarly, a study on an Iranian population observed a correlation between the presence of the restriction site (b allele) and occurrence of breast cancer (Shahbazi *et al*, 2013). The variations observed in results from various studies may be attributed to the number of participants, their race or ethnicity, and various other factors.

The mechanisms underlying breast carcinogenesis may not be well established, however, anti-cancerous functions of vitamin D have been identified in some

malignancies (Iqbal MuN *et al*, 2015). In recent years, a number of studies have hypothesized that VDR polymorphisms may influence both the risk of cancer incidence and its prognosis. Further investigations are required to confirm the role of VDR polymorphisms in preventing breast cancer. The identification of a molecular target and development of a novel treatment method requires further exploration of different polymorphisms in the VDR gene. Simultaneous consideration of the presence of these polymorphisms along with various other genetic and environmental risk factors is essential to identify groups at risk. At present, data from studies investigating these associations mostly show controversial results. It is, therefore, not possible to make definitive statements about the role of *Bsm1* VDR polymorphism in breast cancer occurrence at present. Further research is required to explore the role of these polymorphisms in breast cancer. The assessment of these polymorphisms is essential to identify the groups at risk. The genetic abnormalities and their clinico-pathological significance, if proven, may be used to develop strategies to target it.

## CONCLUSION

Our data did not show any statistical significance at the  $p < 0.05$  level, and shows no evidence of the VDR *Bsm1* polymorphism being associated with breast cancer incidence in the studied population. In conclusion, we did not find any evidence that differences in the VDR gene *Bsm1* alleles confer genetic predisposition to breast carcinogenesis. There were no significant differences in genotype/allele frequencies between cases and controls. The comparison of the patient group with the control group did not reveal any significant increase in risk for breast cancer associated with the VDR *Bsm1* polymorphism.

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