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RESEARCH ARTICLE

Genetic Diversity and Distribution of Vitamin D Receptor (VDR) Genotypes in Breast Cancer Cases from Pakistan

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Abstract

Background: Breast carcinoma is one of the most commonly diagnosed invasive malignancies in females. In Pakistan, it is more commonly detected in women at a young age as compared to the West. Among all women, the risk of developing breast cancer is equal irrespective of their ethnic or racial basis. The aim of the study was to determine vitamin D receptor gene polymorphisms (*FokI* and *TaqI*) and allele frequency distribution in Pakistani women with newly diagnosed breast cancer. This study also aimed to find and compare genetic diversity of VDR polymorphisms among breast cancer cases in different population groups. **Methods:** Newly diagnosed women having breast cancer (n=300) were selected for the study. Blood samples of all the participants were analyzed for vitamin D levels and isolated DNA was subjected to PCR-RFLP analysis. **Results:** Results revealed that allelic frequency of *FokI* and *TaqI* was 'F'; 'f' 50.67 and 49.33% and 'T' and 't' was 46.67 and 53.33 respectively in Pakistani women with breast cancer. The genotypic frequency is significantly ($P<0.05$) distributed. **Conclusion:** The current study concluded significant difference in genotypes and allele frequency of VDR gene polymorphism in Pakistani population suffering from breast cancer when compared with other population.

Keywords: Breast cancer- Vitamin D deficiency- VDR gene polymorphism- *FokI* and *TaqI* genotypes- Pakistan

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Introduction

Breast carcinoma is one of the major invasive malignancies and is listed to be the second globally fatal cancer of women [1]. It is most common in females at a young age in Pakistan as compared to the West where it is most common after the age of 60 years. All women regardless of their ethnic or racial origin or heritage are at risk of developing breast cancer. Different factors contributes in the development of breast cancer such as reproductive experience, genetics and environment, the change in immune status, the effect of endogenous and exogenous hormones in females, host susceptibility and the breast carcinoma's biologic determinants [2]. In Pakistan, almost one in every nine females experience breast cancer and it becomes a maximum rate of occurrence in Asia [3]. Frequency of breast cancer in Pakistani females is 50/100,000. In India, with similar socio-cultural conditions it is 19/100,000. In Pakistan, increase rate of breast cancer development has been seen in premenopausal females and the risk is plateaus after the age of 45 years [3]. Epidemiological evidence suggests that deficiency of vitamin D may be directly associated with incidence of breast cancer [4]. Vitamin D binds to its receptor (VDR) which is present in all body organs and tissues [5]. Our previous study also showed vitamin D deficiency in newly diagnosed breast cancer females [6]. The size of VDR gene is 5.6 kb and is located on chromosome 12 (12q12-14) [7]. The occurrence of genetic polymorphisms in VDR gene has been reported previously and more than 470 Vitamin D receptor SNPs (single nucleotide polymorphisms) have been identified. The two most common previously reported VDR gene's SNPs in Caucasian subjects are rs731236 (*TaqI*) and rs2228570 (*FokI*) [8]. These two restriction fragment length polymorphisms (RFLPs) in the VDR gene were identified by using *FokI* and *TaqI* restriction enzymes. The *TaqI* RFLP is located in exon 9 and have unknown

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function, while the *FokI* RFLP is located on the 2nd exon and caused by nucleotide substitution from T to C [9]. Previous studies indicated that the presence of *ff* genotype was associated with 34% higher breast cancer risk in the nurses' health study and was found to be significant when analyzed statistically [10]. Earlier studies based on smaller number of subjects, investigating association of *FokI* with breast cancer risk were found null [11, 12].

Almost 200 polymorphisms have been previously described in VDR, and among them *Fok1*, *Cdx2*, EcoRV, *Taq1*, *Apa1*, and *Bsm1* have been found to be more frequently associated with tumorigenesis, though there are minor controversies still present in this data [13]. The most frequently reported SNP sites were the polymorphism in start codon of *FokI* and 8th intron of Bsm I among Western and Asian populations. A limited data is available in Pakistan regarding allele frequency distribution and genotype of VDR gene polymorphism in women with breast cancer. Therefore, the present study is an attempt to investigate the distribution of VDR gene polymorphism (*FokI*, *TaqI*) in Pakistani women with breast cancer by using Polymerase Chain Reaction (PCR) based restriction analysis and to compare genotype frequency with different populations suffering from breast cancer.

Materials and Methods

Three hundred newly diagnosed breast cancer females visiting Institute of Nuclear Medicine and Oncology Lahore (INMOL) from different areas of province Punjab, Pakistan have been included in this study. Age of patients was between 19-75 years. A consent (in writing) was taken from each female participating the study. The study approval was obtained from the scientific research review and ethical committee of the institution. The committee approved the study protocol including biochemical estimations, and VDR gene SNP analysis in the study participants. Five ml of blood samples were collected by venipuncture for biochemical estimations and isolation of DNA.

DNA extraction

The genomic DNA was isolated from blood of patients using DNA extraction kit, (Vivantis GF-1, Germany). The absorbance (OD) at 260/280 nm was measured for the determination of concentration and purity of extracted DNA. DNA samples were stored at -20 °C for further analysis.

VDR Genotyping

PCR amplification of VDR gene was done using primer sequences published previously [14, 15] in 25µl reaction mixtures containing 25ng of genomic DNA and 5pmol each primers for *FokI*/*Taq1* polymorphism in (thermal cycler (Mastercycler gradient, Germany). The PCR conditions (*Fok1*) were initial denaturation at 94 °C for 5 minutes, followed by 35 cycles at 94 °C for 30 seconds, 58 °C for 30 seconds and 72 °C for 1 minute and one final cycle of extension at 72 °C for 7 minutes [14]. The conditions for PCR amplifications (*Taq1*) was initial denaturation at 94 °C for 3 minutes followed by 35 cycles at 94 °C for 30 seconds, 62.8 °C for 35 seconds, 72 °C for 45 seconds and final extension at 72 °C for 10 minutes [15]. The amplified PCR product (265bp/465bp) was digested with 1.0 unit of *FokI*/*Taq1* restriction enzyme (Thermo Scientific, Fast Digest enzyme) at 37 °C for 5 minutes. The 10µl reaction mixture after digestion was run on agarose gel (2%) containing ethidium bromide. Genotypes were assigned on the basis of digested product length as: FF homozygous for the absence of the *FokI* restriction site with an undigested band of 265 bp; ff homozygous for the presence of the *FokI* restriction site with complete digestion resulting in 196bp and 69bp bands and Ff heterozygous with all three bands (265 bp, 196bp and 69bp). *TaqI* restriction site as TT homozygous for the absence of *TaqI* site with an intact 454 bp band, Tt heterozygous with three bands (454bp, 293bp and 161bp) and tt homozygous with 2 bands of 293bp and 161bp.

Statistical Analysis

Genotype and allelic frequencies of the VDR gene polymorphisms (*FokI* and *TaqI*) in breast cancer patients was determined by Hardy-Weinberg equilibrium. P value <0.05 was considered significant for the data (level of significance was kept 5%).

Results

The present study was conducted on 300 newly diagnosed breast cancer women. Among these females, 55% were premenopausal (n=164) and 45% were postmenopausal (n=136). The mean ± SD age and BMI of breast cancer women was 44.0±10.9years and 25.6±4.5 kg/m^2 respectively. The data showing frequency of clinical features in studied patients was shown (Table 1).

Genotype analysis of *FokI* and *TaqI* SNPs was completed by PCR based restriction analysis. The bands on the 2% agarose gel showed undigested (FF), digested

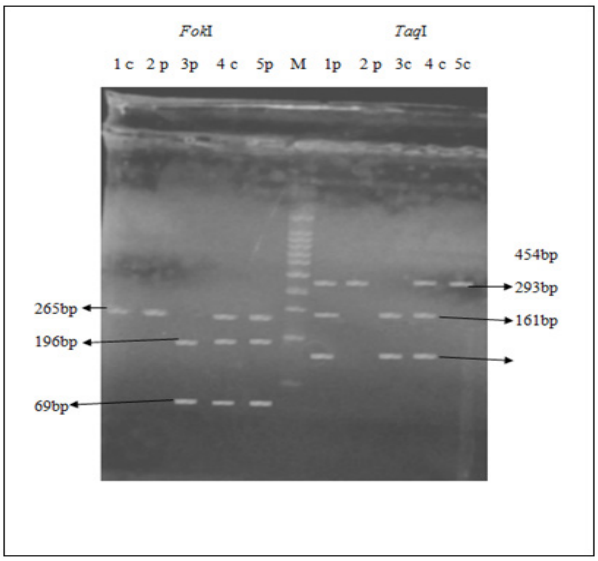

Figure 1. PCR-RFLP of VDR Gene Showing Restriction Sites of Taq1 and Fok1 SNPs

Table 1. VDR Genotype Frequencies and Clinico-pathological Features of Breast Cancer Patients.

Features	Number of cases	Genotype frequency for *FokI* SNP			Genotype frequency (N) for *TaqI* SNP		
		FF (%)	Ff (%)	ff (%)	TT (%)	Tt (%)	tt (%)
		N=137	N=30	N=133	N=60	N=80	N=160
Menopausal status							
Pre	166	80 (58)	17 (57)	69 (52)	45 (75)	34 (42)	87 (54)
Post	134	57 (42)	13 (43)	64 (48)	15 (25)	46 (58)	73 (46)
P-value			0.554*			0.001*	
Tumor Type							
IDC	264	119 (87)	22 (73)	123 (93)	49 (67)	70 (88)	145 (91)
ILC	36	18 (13)	8 (27)	10 (7)	11 (33)	10 (12)	15 (9)
P-value			0.012*			0.188*	
Tumor grade							
I	40	11 (8)	11 (37)	18 (14)	12 (20)	10 (12)	18 (11)
II	142	55 (40)	1 (3)	86 (65)	18 (30)	30 (38)	94 (59)
III	92	61 (45)	4 (13)	27 (20)	17 (28)	35 (44)	40 (25)
IV	26	10 (7)	14 (47)	2 (1)	13 (22)	5 (6)	8 (5)
P-value			<0.001*			<0.001*	
ER/PR							
+/+	98	61 (37)	13 (44)	24 (18)	50 (83)	18 (22)	30 (19)
-/-	106	2 (1)	10 (33)	94 (71)	1 (2)	23 (29)	82 (51)
+/-	64	60 (37)	1 (3)	3 (2)	4 (7)	30 (38)	30 (19)
-/+	32	14 (25)	6 (20)	12 (9)	5 (8)	9 (11)	18 (11)
P-value			<0.001*			<0.001*	
Her-2							
+ve	140	74 (54)	20 (67)	46 (35)	22 (37)	39 (49)	79 (49)
ve	160	63 (46)	10 (33)	87 (65)	38 (63)	41 (51)	81 (51)
P-value			<0.001*			0.221*	

*Chi^2 test

(ff) and heterozygotes (F/f) (Figure 1). The VDR gene sequence of amplified product for *FokI* polymorphism was submitted in GenBank with accession no. MH064431.

The clinical features and genotypic frequency distribution of VDR gene polymorphisms (*FokI*, *TaqI*) SNPs with respect to menopausal status, tumor type, grade and hormonal status was shown in Table 1. The *TaqI* genotypic frequency distribution was found statistically significant for menopausal status, tumor grade ($p < 0.05$) (Table 1) whereas *FokI* genotypic frequency distribution vary significantly ($p<0.05$) for tumor type, tumor grade and ER/PR/Her2 status (Table 1).

Distribution of VDR Genotypes

The frequencies (%) of alleles and genotypes *TaqI* (rs731236) and *FokI* (rs2228570) SNPs in Pakistani women with breast cancer were shown (Table 2). The allelic frequencies of 'F' vs 'f' and 'T' vs 't' were 50.67 vs 49.33% and 46.67vs 53.33% respectively in Pakistani population. Out of 300 breast cancer patients analyzed, a significant allele and genotype distribution frequency of VDR polymorphisms for *TaqI* rs (731236) and *FokI* rs (2228570), was observed in this study ($p<0.00001$). A significant difference in genotype frequency of these SNPs in breast cancer patients was observed on comparison with other reported populations (Table 3). The minor allele 'f' of *FokI* was highly prevalent in our patients as compared to patients of European and Asian origin (Table 3).

Table 2. Genotypes and Allele Frequency of VDR Gene (*FokI*, *TaqI*) Polymorphism in Newly Diagnosed Breast Cancer Women from Pakistan

Genotypes n=300 (%)			Allelic frequencies (%)		
FF	Ff	ff	F	f	p-value
137 (46)	30 (10)	133 (44)	50.67	49.33	<0.0001
TT	Tt	tt	T	t	
60 (20)	160 (53)	80 (27)	46.67	53.33	<0.0001

Table 3. Comparison of VDR Genotype Frequency Distribution among Breast Cancer Patients from Pakistan and other Studied Populations

			*Fok*I gene polymorphism			
Population	Sample size	FF (%)	Ff (%)	ff (%)	X^2	*p*
Uk-Caucasian [11]	181	39.8	44.8	15.5	0.43	0.511
German [17]	1390	40.7	43.6	15.7	6.762	0.009
French-Canadian [19]	859	33.8	47.8	18.4	0.3412	0.559
Iranian [20]	95	16.8	8.4	74.7	90.07	<0.00001
Present study	300	46	10	44	191.98	<0.00001
			TaqI gene polymorphism			
Population	Sample size	TT (%)	Tt (%)	tt (%)	X^2	*P*
Turkish [21]	78	33.3	56.4	10.3	2.865	0.09
German [17]	1403	35.4	47.6	17	0.354	0.551
Jordanian [22]	122	41	45.9	13.1	0.0027	0.958
Present study	300	20	53	27	48	<0.00001

Discussion

In numerous studies, VDR polymorphism have been reported by using Restriction Fragment Length Polymorphism (RFLP) assay to conclude ethnic differences [16]. These ethnic differences arise from the genotypes and alleles frequency variations. In this study, the genotypes and allele frequency of VDR (*Fok-1* and *Taq-I*) were reported in Pakistani population suffering from breast cancer and compared with those reported in different studies worldwide as shown previously [17, 11]. The genotype frequency of VDR gene polymorphisms in Pakistani healthy women population has been reported [18] but still no data is available regarding distribution of these SNPs in Pakistani women with newly diagnosed breast cancer. The study presented here is the first report to describe the receptor gene polymorphisms (*FokI*, *TaqI*) of vitamin D in newly diagnosed breast cancer women. In our study, correlation between *FokI* and *TaqI* genotypes frequencies with different clinicopathological features was also studied. The distribution of *FokI* genotypic frequency was found to be nonsignificant (p=0.554) for menopausal status and tumor type. However, for *TaqI* SNP it was significantly different (p=0.001) between pre and post menopausal groups (Table 1). Both *FokI* and *TaqI* genotypic frequency distribution vary significantly (P=<0.001) among women with different tumor grade and ER/PR/Her2 status except for *Taq1* where it was found nonsignificant for Her2 status (p=0.221). Significant differences between *Fok-I* genotypes frequencies regarding family history of BC, type and grade of tumor was reported in other studies whereas no significant differences were found with respect to age, menopausal status, ER/PR status and Her-2 status in the same study .

The genotype frequency was compared with different studies reported worldwide (Table 3). The allelic frequency (Table 2) of 'F'; 'f' was 50.67 and 49.33% and 'T' and 't' was 46.67 and 53.33% (p= <0.00001) respectively in this study. Our results revealed a significant difference in genotype and allele frequency of VDR gene polymorphisms (*FokI* and *TaqI*) in Pakistani women with breast cancer. The study also revealed that the genotype distribution of the *Fok-1* (rs2228570) in the Pakistani population is significantly different as compared to the Caucasians and European populations [11]. In an Asian population of Iranian women, suffering from breast cancer, similar significant genotype distribution of *Fok-I* polymorphism F=14.7 and f=85% was found [19]. Likewise, Taq (rs731236) allele and genotype frequencies in Pakistanis were significantly different from other Asian populations (Table 3). These differences in genotype frequencies highlights the role of ethnicity according to the geographic distributions as reported earlier. It has also been reported that the frequency of minor allele of *FokI* (rs2228570) was considerably lower in Africans compared to Asians or Caucasians.

The "ff genotype" frequency has been previously reported as 4% in African Americans and approximately 13-18 percent among Asians and Caucasians [20] whereas in Asians the minor allele of *TaqI* rs731236 was found to be present in much lower frequency than in Caucasians and Africans [16]. Although VDR polymorphisms may influence disease risk, such genetic factors cannot be dissociated from environmental influences that may be potential influential factor of such genetic variations [16].

In conclusion, the present study concluded that Pakistani females with breast cancer were relatively younger as compared to other reports with median age of 44 years. The common clinical features in our patients with breast cancer include invasive ductal carcinoma, grade II tumor and negative hormonal status (ER/PR, Her2). Significant difference in genotypes and allele frequency of VDR gene polymorphism in Pakistani females with breast cancer (taken as an independent ethnic group) was observed when compared with other populations in terms of ethnic groups. These results showed the impact of ethnicity on VDR polymorphism distribution in our patients as well as other ethnicities which were categorized into American, African, Asian and Caucasian. Moreover, possible role of ethnic as well as geographic differences in genetic profile variation together with other factors such as living environment,

dietary habits and lifestyle needs to be considered as well. Allelic association studies are underway to understand the relationship between the VDR gene polymorphisms and breast cancer risk and whether these SNPs can be used as predictive markers of genetic susceptibility in our population.

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Author Disclosures

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