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ORIGINAL ARTICLE

Vitamin D Receptor Gene Polymorphism: Association with Susceptibility to Early-Onset Breast Cancer in Iranian, *BRCA1/2*-Mutation Carrier and non-carrier Patients

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Abstract Mounting evidences support that vitamin D insufficiency or deficiency is a risk factor of breast cancer. Vitamin D receptor (VDR) is expressed in more than 36 cell types in different organs as in cancerous cells. Numerous allelic variants of VDR gene have been identified in human populations. Association of *FokI* (rs2228570) and *BsmI* (rs1544410) single nucleotide polymorphisms (SNPs) in *VDR* gene with the risk of breast cancer have been investigated in several studies, however, the published data are still inconsistent. Here, we investigated *BsmI* and *FokI* polymorphisms in Iranian young (≤ 35 years old) breast cancer patient with known *BRCA1/2* germline mutations. *VDR* gene polymorphisms were detected by restriction fragment length polymorphism (RFLP) analysis in a cohort of 203 breast cancer patients and 214 controls from Iran. There was a significant association between the bb and Bb genotypes of the *BsmI* and the increased risk of breast cancer (OR 1.74, CI 1.06–2.87 and OR 2.08, CI 1.31–3.29, respectively). This association was maintained in the subgroup of *BRCA1/2* mutation non carriers (OR 1.90, CI 1.15–3.20 and OR 1.75, CI 1.07–2.87 for bb and Bb genotypes respectively) and in the subgroup of BRCA1/2 mutation non-carriers with a family history of breast and/or ovarian cancer (OR 1.81, CI 1.08–3.05 and OR 1.65, CI 1.00–2.70 for bb and Bb genotypes respectively). None of the *FokI* homozygous or heterozygous genotypes were associated with the risk of breast cancer. In summary, the *BsmI* polymorphism of *VDR* gene may be associated with the risk of breast cancer in Iranian women.

Keywords Breast cancer · Vitamin D receptor · Polymorphism

Introduction

Accounting for 24.6% of all malignancies, breast cancer is the most common cancer in Iranian females. Incidence of breast cancer increased significantly in Iran in past decade with mean age of 10 years less than developed countries [1]. A set of environmental and genetic factors contribute to the development of breast cancer. Among the recognized genes related to this malignancy, *BRCA1* and *BRCA2* are undoubtedly the most important genes. Some mutations in these genes increase the risk of breast cancer in carriers to more than 50% [2]. Mutation in genes encoding tumor protein (TP53), phosphatase and tensin homolog (PTEN), checkpoint kinase 2 (CHEK2) and radiation protein 51C (RAD51C) has also been associated to breast cancer development [3, 4].

Mounting evidences in recent years, support that 1,25 (OH)2 vitamin D3 insufficiency or deficiency could be a risk factor of breast cancer. This could be explained by the fact that vitamin D is not only an endocrine hormone contributing to the calcium homeostasis but also participate in fundamental cellular process including apoptosis, cell growth and proliferation [5]. Vitamin D induces its function by binding to and activation of vitamin D receptor (VDR). VDR is expressed in

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more than 36 cell types in different organs as in cancerous cells. Upon activation by 1,25 (OH)2 vitamin D3, VDR forms heterodimer with retinoid X receptor and binds to the hormone response elements in target genes [6]. *VDR* gene size is 75 kilobase that contains 14 exons; DNA binding domain of VDR is encoded by exons IA-IC along with exon II and exon III and ligand binding domain is encoded by exons IV-IX. The 5′untranslated region is related to exons ID-IF [7]. It is reported that *VDR* gene expression decreases in breast cancer cells. Therefore, vitamin D signaling pathways may repress in these cells due to altered expression of *VDR* gene even in individuals with normal or high vitamin D levels [8].

Numerous allelic variants of *VDR* gene have been identified in human populations that may be a cause of decreased expression of *VDR* in breast cancer cells and also affect *VDR* mRNA stability [9]. *FokI* (rs2228570) and *BsmI* (rs1544410) are among the most common single nucleotide polymorphisms (SNPs) in *VDR* gene. The *FokI* SNP is located in exon II and is associated with VDR protein length. F allele is more frequent than f and encodes a protein that lacks first 3 N-terminal residues. The *BsmI* SNP is located on intron VIII and there is no evidence indicating the effect of this SNP on VDR protein structure or its expression, however, there is linkage disequilibrium between this SNP and polyA sequence within the 3′-untranslated region (3′-UTR) that may affect *VDR* mRNA stability [7].

Association of vitamin D receptor gene polymorphisms with breast cancer risk have been investigated in several studies, however, the published data are still inconsistent [10]. Most of these studies were conducted on Caucasians and a few on Asians. *BsmI* SNP was associated with breast cancer in Pakistani women negative for *BRCA1/2* mutations [11] and also Japanese-American [12] and Iranian women [13], whereas no association was found in Chinese women [14]. *Fok1* SNP showed an association with breast cancer in Japanese-American women [12], and no association in Chinese [14], Pakistani [11], or Iranian women [13]. Mutation in other susceptibility genes such as *BRCA1/2* may be a cause of these inconsistent results. Sampling in a wide range of ages and different menopausal status of samples are other possible reasons of these controversial results. To remove these confounding factors, here, we investigated *BsmI* and *FokI* polymorphisms in Iranian young (≤ 35 years old) breast cancer patient with known *BRCA1/2* germline mutations.

Materials and Methods

Patients and Samples

Between November 2008 and April 2011, a total of 203 breast cancer patients were enrolled in this study at Shahid Rajayee hospital, located Babolsar, north of Iran. All the subjects were 35 years old or younger at diagnosis and *BRCA1*/2 germline mutations were screened in all the patients. Deleterious mutations in *BRCA1*/2 genes were identified in 42 patients (A. Shahabi, unpublished data). The following information were collected from pathology medical records: histology (ductal, lobular and other), hormone receptor status, human epidermal growth factor receptor 2 (Her2) status, lymph node status, tumor size and histological grade. Moreover 214 healthy gender and age matched volunteer with no prior history of cancer and no family history of breast cancer were enrolled in this study. A peripheral blood sample was collected after receiving informed consent and agreement from both patients and volunteers. The study was confirmed by the ethics committee of our organization.

Extraction of Genomic DNA and Genotyping

Blood samples were collected in EDTA-treated tubes for DNA extraction. We used DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) for extraction of genomic DNA from whole blood according to manufacture's instruction. The extracted genomic DNA was stored at -70 °C in 200 μl elution buffer for further analysis. *VDR* genotyping for *FokI* and *BsmI* was done by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. For amplification of *FokI* polymorphic site the following forward 5′-CTGGCACTGACTCTGGCTCT -3′ and reverse 5′-GGGCTCACCTGAAGAAGCCT -3′ primers and for *BsmI*, the primers forward 5′-CCTCACTGCCCTTAGCTCTG-3′ and reverse 5′-TCTCACCTCTAACCAGCGGA-3′ were used [13]. For *BsmI*, each reaction mixture was amplified for 40 cycles as follows: 94 °C (30 s), 59 °C (30 s) and 72 °C (30 s) and for *FokI* 40 cycles as follows: 94 °C (30 s), 59.5 °C (30 s) and 72 °C (30 s). A pre-incubation of 5 min at 94 °C and a final extension for 7 min at 72 °C were performed in both PCRs. In the next step, PCR products were digested with BsmI and FokI according to manufacture's instruction (Thermo Fisher Scientific, Waltham, MA, USA) and fragments were separated with 2% agarose gel electrophoresis containing ethidium bromide. A single 290 bp fragment on the gel indicated that there was no recognition site for BsmI (BB homozygotes), two fragments of 181 and 109 bp was indicator of two recognition site (bb homozygotes) and all the three mentioned fragments were representative of Aa heterozygous. For the *FokI*, FF homozygous was recognized by a 204 bp fragment, ff homozygotes by 50 and 154 bp fragments and Ff heterozygous by 273, 197, and 76 bp fragments.

Statistical Analysis

Chi-square test was used to evaluate if the genotyped *VDR* SNPs were consistent with Hardy-Weinberg equilibrium (HWE). Association of each SNP with breast cancer risk

was estimated by Logistic Regression test at 95% confidence interval, and adjusted for the following breast cancer risk factors: Age, age at first FTP, number of pregnancies, breastfeeding, menarcheal age, BMI and smoking. [11]. All the cases were stratified according to *BRCA1/2* mutation status and *BRCA1/2* mutation carriers and non-carriers were separately compared to the control group. In addition, patients that did not carry a *BRCA1/2* mutation were divided into two subgroups according to family history of breast and/or ovarian cancer in their first-degree relatives and separately compared to the control group. Both SNPs were analyzed regarding their correlation with histopathological tumor characteristics including estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2/neu) status, tumor grade and size, nodal status and morphology, using logistic regression. For analysis of the association between *VDR* SNPs and breast cancer risk, the more prevalent genotype i.e. BB and FF were assigned as reference genotype. Several previous studies used these genotypes as reference too [11, 13, 15]. All statistical analyses were performed using IBM® SPSS® Statistics version 20 for Windows (SPSS Inc., Chicago, Illinois). $P < 0.05$ was indicative of statistical significance.

Results

Characteristics of the Study Participants

This case control study consisted of 203 breast cancer cases with known *BRCA1/2* mutation status and 214 controls from Iran. The median age of cases at diagnosis was 31 years (range 19–35 years) and the median age of controls at study entry was 30 years (range 18–35 years). From the control group 87% were healthy, while, 4% suffered from diabetes type I or II, 4% from hypertension, 3% from asthma or allergy and 2% from hepatitis B. The characteristics of cases and controls are depicted in Table 1. When risk factors associated with breast cancer were compared between cases and controls it was observed that except number of pregnancies ($P = 0.01$) and history of breast feeding ($P = 0.02$) there was no significant difference in other parameters.

The Frequencies of *FokI* and *BsmI* SNPs of *VDR* Gene and their Association with Breast Cancer Risk

In this study *BsmI* and *FokI* SNPs of *VDR* gene were detected after digestion of PCR products by related restriction enzymes. The distribution of genotypes for the *FokI* SNP were consistent with HWE in both the cases ($P = 0.52$) and controls ($P = 0.56$). The frequency of the f allele of the *FokI* among the cases and controls was 0.26 and 0.28 respectively. Most of the previous studies reported higher frequencies of this SNP. However in the only study in Iran similar values were reported [13]. Statistical analysis revealed that *FokI* ff genotype was not associated with the risk of breast cancer in our investigated population (OR 1.19, 95% CI 0.58–2.45, $P = 0.62$). The same result was observed for the *FokI* heterozygous genotype (OR 1.06, 95% CI 0.50–2.20, $P = 0.87$). None of the *FokI* homozygous or heterozygous genotypes were associated with the risk of breast cancer in the subgroups of *BRCA1*/2 mutation carriers and non-carriers (Table 2) and also in the subgroups of *BRCA1*/2 mutation non-carriers with and without a family history of breast and/or ovarian cancer (Table 3).

No deviation from the expected HWE was observed for the *BsmI* SNP in both the case ($P = 0.49$) and control groups ($P = 0.51$). The frequency of the b allele of the *BsmI* was 0.47 and 0.43 among the cases and controls respectively. Statistical analysis revealed that there was a significant association between the *BsmI* bb genotype and the increased risk of breast cancer (OR 1.74, 95% CI 1.06–2.87, $P = 0.02$). This association was maintained and even stronger in the subgroup of *BRCA1/2* mutation non carriers (OR 1.90, 95% CI 1.15–3.20, $P = 0.01$) (Table 2) and in the subgroup of *BRCA1/2* mutation non-carriers with a family history of breast and/or ovarian cancer (OR 1.81, 95% CI 1.08–3.05, $P = 0.02$) (Table 3). An association between *BsmI* Bb genotype and increased risk of breast cancer was also observed (OR 2.08, 95% CI 1.31–3.29, $P = 0.002$) and maintained in the subgroup of *BRCA1/2* mutation non carriers (OR 1.75, 95% CI 1.07–2.87, $P = 0.03$) and in the subgroup of *BRCA1/2* mutation non-carriers with a family history of breast and/or ovarian cancer (OR 1.65, 95% CI 1.00–2.70, $P = 0.04$).

Histopathological Characteristics of Breast Tumor and their Association with *VDR FokI* and *ApaI* SNPs

The clinicopathological features of breast cancer patients are presented in Table 4. Most of the cases were affected by ductal carcinoma (91%) and a few with lobular carcinoma (4%) and other types (5%). ER was highly expressed in 41% of the cases, PR in 38% and HER2/neu in 33% of the patients. For finding any possible association between investigated SNPs and tumor progression, the correlation of *BsmI* and *FokI* SNPs with histopathological tumor characteristics were analyzed. There was no association between these SNPs and the investigated tumor parameters (data not shown).

Discussion

Genetic and environmental factors and also interactions between them contribute to the development of breast cancer. Accumulated data have suggested that 1,25 (OH)2 vitamin D3 insufficiency or deficiency could be a risk factor of breast cancer [5]. All the wide effects of this vitamin on the cell

Table 1 Characteristics of breast cancer patients and controls

Clinical Characteristic	Cases ($N = 203$) n (%)	Controls ($N = 214$) n (%)	P-value[1]
Age at diagnosis/recruitment (years)			0.10
< 20	4 (2)	13 (6)	
20–29	75 (37)	73 (34)	
30–35	124 (61)	128 (60)	
Age at menarche (years)			0.34
< 12	16 (8)	24 (11)	
12–14	134 (66)	128 (60)	
> 14	53 (26)	62 (29)	
Age at first FTP (years)			0.40
< 20	28 (14)	36 (17)	
20–24	43 (21)	66 (31)	
25–29	57 (28)	58 (27)	
> 29	16 (8)	26 (12)	
Number of pregnancies (≥28th week)			**0.01**
0	59 (29)	28 (13)	
1	69 (34)	81 (38)	
2	57 (28)	75 (35)	
3	18 (9)	26 (12)	
≥ 4	0 (0)	4 (2)	
Ever breastfeeding			**0.02**
Yes	130 (64)	167 (78)	
No	73 (36)	47 (22)	
O.C use			
Yes	168 (83)	169 (79)	0.38
No	35 (17)	45 (21)	
BMI (kg/m^2)			
Normal (18.5–24.9)	79 (39)	92 (43)	0.61
Underweight (<18.5)	39 (19)	36 (17)	
Overweight (25–29.9)	65 (32)	60 (28)	
Obes (≥30)	20 (10)	26 (12)	
Smoking			
Yes	6 (3)	4 (2)	0.53
No	197 (97)	210 (98)	

[1] Based on two-sided Chi-square test

P-values below 0.05 are marked in bold

growth, division and differentiation are mediated by binding of this molecule to its receptor i.e. VDR. Therefore factors that influence *VDR* gene expression and also different polymorphisms of this gene and their association with the risk of breast cancer has attracted a lot of attention. In this study, *VDR FokI* and *ApaI* polymorphisms and their correlation with the risk of breast cancer have been explored. The main difference of this study from many others is that *BRCA1/2* mutation status of patients was clear to us. The data presented in Table 2 clearly indicate the importance of knowing *BRCA1/2* mutation status. The *BsmI* SNP had a significant association with increased risk of breast cancer, however, this association was only confined in the subgroup of *BRCA1/2* mutation non-carriers and no association was observed in the subgroup of *BRCA1/2* mutation carriers. These findings could be interpreted by the fact that *BRCA1* and *BRCA2* are high penetrance genes associated with increased risk for breast cancer. As about 10% of breast cancer incidences are due to the mutation in *BRCA1* and *BRCA2* genes, considering these genes and removing *BRCA1/2* mutation carriers from the case group improved the power of our case-control comparison.

In this study that included 203 cases and 214 controls, it was observed that the risk of breast cancer was not affected by none of the genotypes and alleles related to the *VDR FokI*

Table 2 Association of *FokI* and *BsmI* SNPs with breast caner

	Controls	Cases								
	(*N* = 214)	All the cases (*N* = 203)			*BRCA1/2* mutation carriers (*N* = 42)			*BRCA1/2* mutation non-carriers (*N* = 161)		
Genotype	n (%)	n (%)	OR (95% CI)	*P*-value	n (%)	OR (95% CI)	*P*-value	n (%)	OR (95% CI)	*p*-value
FokI										
ff	19 (9)	16 (8)	1.19 (0.58–2.45)	0.62	5 (11)	0.68 (0.22–2.04)	0.49	11 (7)	0.89 (0.44–1.80)	0.75
Ff	84 (39)	75 (37)	1.06 (0.50–2.20)	0.87	17 (41)	0.77 (0.25–2.34)	0.64	58 (36)	0.64 (0.30–1.33)	0.65
FF	111 (52)	112 (55)	1 (reference)	-	20 (48)	1 (reference)	-	92 (57)	1 (reference)	-
BsmI										
bb	47 (22)	55 (27)	1.74 (1.06–2.87)	**0.02**	5 (26)	1.01 (0.63–1.62)	0.80	50 (31)	1.90 (1.15–3.20	**0.01**
Bb	58 (27)	81 (32)	2.08 (1.31–3.29(	**0.002**	25 (25)	0.99 (0.62–1.60)	0.83	56 (35)	1.75 (1.07–2.87)	**0.03**
BB	109 (51)	67 (41)	1 (reference)	-	12 (49)	1 (reference)	-	55 (34)	1 (reference)	-

P-values below 0.05 are marked in bold

SNP. This lack of correlation was observed again in the subgroup of *BRCA1/2* mutation carriers and non-carriers and also when patients where stratified according to family history of breast and/or ovarian cancer. Many studies to date investigated the association between *VDR FokI* SNP and the risk of breast cancer. Most of the studies on Caucasians found significant association between the ff genotype and the risk of breast cancer and meta-analysis studies also confirmed this association [10, 16]. A few studies investigated this SNP in Asians. In a comprehensive study in china that included 2919 cases and 2323 controls no association between *FokI* SNP and the risk of breast cancer was observed [14]. This lack of association was observed again in a Pakistani population including 463 cases and 1012 controls [11]. In the only study conducted in Iran, Shahbazi et al. [13] investigated 140 cases and 156 controls and found no association between *FokI* SNP and the risk of breast cancer. Similar results were observed in other ethnicities such as Hispanics and African-Americans [17]. However, investigation of Japanese-American women reveled an association between ff genotype and increased risk of breast cancer [12].

In this study there was a significant association between the *b* allele of the *BsmI* and increased risk of breast cancer. Further statistical analysis revealed that this association is confined to the subgroup of *BRCA1/2* mutation non-carriers. Meta-analysis of 31 studies revealed no significant association between this allele and the risk of breast cancer in Caucasians [18]. But the results of the limited number of studies on Asians are controversial. In a large study in China, no meaningful association between the *b* allele of the *BsmI* and the risk of breast cancer was found [14]. However, in two studies in Iran [13] and Pakistan [11] a correlation was observed. In Pakistani

Table 3 Association of FokI and BsmI SNPs and breast cancer in *BRCA1/2* mutation non-carriers with and without family history of breast and/or ovarian cancer

	Controls	Cases					
	(*N* = 214)	With family history of breast and/or Ovarian cancer (*N* = 45)			without family history of breast and/or Ovarian cancer (*N* = 116)		
Genotype	n (%)	n (%)	OR (95% CI)	*p*-val ue	n (%)	OR (95% CI)	*p*-value
FokI							
ff	19 (9)	5 (11)	0.95 (0.46–1.97)	0.90	6 (5)	1.31 (0.57–2.96)	0.51
Ff	84 (39)	17 (38)	0.82 (0.42–1.60)	0.56	41 (35)	0.82 (0.49–1.36)	0.44
FF	111 (52)	23 (51)	1 (reference)	-	69 (60)	1 (reference)	-
BsmI							
bb	34 (16)	19 (42)	1.81 (1.08–3.05)	**0.02**	31 (27)	1.54 (0.91–2.61)	0.22
Bb	118 (55)	10 (22)	1.65 (1.00–2.70)	**0.04**	46 (40)	1.40 (0.85–2.32)	0.18
BB	62 (29)	16 (36)	1 (reference)	-	39 (33)	1 (reference)	-

P-values below 0.05 are marked in bold

Table 4 Clinical characteristics of breast tumors

Clinical Characteristic	Cases ($N = 203$) n (%)
Histological type	
Ductal	185 (91)
Lobular	8 (4)
Other types	10 (5)
Tumore Size	
T0	6 (3)
T1	41 (20)
T2	91 (45)
T3	47 (23)
T4	18 (9)
Lymph node status	
N0	87 (43)
N1	116 (57)
Histological grade	
GI	2 (1)
GII	47 (23)
GIII	146 (72)
GIV	8 (4)
HER2/neu status	
Positive	67 (33)
Negative	136 (67)
Estrogen receptor status	
Positive	83 (41)
Negative	120 (59)
Progesterone receptor status	
Positive	77 (38)
Negative	126 (62)

population, like our study, this correlation was confined to the subgroup of *BRCA1/2* mutation non-carriers. A possible explanation is that pathways that lead to breast cancer, suppress in *BRCA1/2* mutation carriers. Studies in other ethnicities did not have consistent results too. No association between *BsmI* alleles or genotypes and the risk of breast cancer was observed in African-American women [19], while in Hispanic ethnicity there was an association between B allele and increased risk of breast cancer [20]. However, other studies on Hispanic women reported no association [12, 17].

Limited sample size in most of the mentioned studies, sampling in a wide range of ages, different menopausal status of samples and different ethnicities of the studied populations are the main possible reasons of these controversial results. In this study all the cases are Iranian and below 35 years old. One of the main strengths of this study is that family history of cancer was considered in statistical analysis. The chance of carrying alleles that increase the risk of cancer, in patients with family history of cancer is more than unselected cancer patients, which was confirmed in this study too. On the other hand, knowing *BRCA1/2* mutation status, allowed us to more accurately relate the risk of breast cancer to *VDR* gene polymorphism. However this study had some limitations too. Vitamin D level and its possible correlation with *VDR* SNPs and their combination in determining the risk of breast cancer was not investigated in this study. On the other hand, environmental factors like duration of exposure to sunlight and dietary factors and their possible correlation with breast cancer risk were not considered in this study. For the future studies investigation of other genes involved in vitamin D processing and their association with the risk of breast cancer are suggested.

In summary, in this study bb and Bb genotypes of *BsmI* were associated with increased risk of breast cancer in the investigated population. Obviously, to confirm these results, more studies in larger populations in Asians are needed.

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Compliance with Ethical Standards

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Conflict of Interest The authors declare that they have no conflict of interest.

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