Journal of BUON **16:** 640-645, 2011

ORIGINAL ARTICLE

Vitamin D receptor gene polymorphisms and male breast cancer risk in Turkish population

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Summary

Purpose: *Male breast cancer (MBC) is a rare disease. However, as global populace ages, there is a trend for MBC increase. Although its etiology is still unclear, constitutional, environmental, hormonal (abnormalities in estrogen/androgen balance) and genetic (positive family history, Klinefelter syndrome, mutations in BRCA1 and BRCA2) risk factors are already known. One potential target is the vitamin D receptor (VDR). We have investigated whether polymorphisms in the VDR gene are associated with altered MBC risk in a Turkish population.*

Methods: *We recruited 25 men with known breast cancer and 96 men selected from blood donations. Polymorphic sites in VDR gene ApaI (rs7975232), TaqI (rs731236) and FokI (rs10735810) were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis.*

Results: *The unconditional logistic regression analysis demonstrated no significant association for the VDR ApaI (p=0.70), TaqI polymorphism (p=0.88) and FokI polymorphism (p=0.075).*

Conclusion: *Our results do not support potential effects of VDR polymorphisms on MBC risk and possible differential effects of receptor status of the tumor. However, further studies focusing on the influence of polymorphisms and haplotypes on VDR functionality, activity and concentration are needed.*

Key words: male breast cancer, polymorphisms, RFLP, vitamin D receptor

Introduction

MBC is a rare disease and little is known about its etiology compared with female breast cancer. MBC represents < 1% of all cancers in men and its incidence is increasing in younger men [1]. In Turkey, MBC accounts for 0.2% of all cancers in males [2]. Breast cancer is known to be strongly influenced by the hormonal milieu, and variations in genes that are responsive to such hormones are therefore possible candidates for increased risk. One potential target is the VDR, a member of the steroid-hormone family of nuclear receptors, which are responsible for the transcriptional regulation of a number of hormone-responsive genes. VDR is expressed in breast tissue, and patients with VDR-positive breast tumors have longer disease-free survival compared to those with receptor-negative tumors [3]. The VDR ligand is the vitamin D metabolite, 1,25 dihydroxyvitamin D3 (1,25-D), which has potent effects on cell growth and differentiation. Laboratory studies have demonstrated that 1,25-D and its analogues inhibit cell proliferation and promote apoptosis in cultured cells [4]. This anticancer potential results from the role of vitamin D as a transcription factor that regulates cell growth, differentiation, and apoptosis, which are cellular mechanisms central to the development and progression of cancer. Moreover, epidemiologic studies suggest an inverse association between sun exposure, serum levels of 1,25-D, and vitamin D intake and the risk of developing and/or surviving cancer [5]. Because this vitamin exerts its function on engagement of its receptor (i.e., VDR), it is likely that polymorphisms of this nuclear hormone receptor family member affect the ability of interacting with its ligand, which ultimately would lead to different levels of vitamin D biologic activity [6]. Several studies have assessed associations between various polymor-

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Received 18-03-2011; Accepted 16-04-2011

phisms in the VDR gene and breast cancer risk, with inconsistent results. These polymorphisms include two frequently analysed, highly linked single nucleotide polymorphisms (SNPs) ApaI rs7975232, TaqI at the 3' end of the VDR gene. The t allele of the TaqI SNP in exon 9 (rs731236, T/C, C = t), which leads to a silent codon change, has been found in different studies to be associated with a nonsignificantly increased breast cancer risk [7] and with a decreased risk for breast cancer [8], or there was no association [9]. Another promising functional polymorphism in the start codon at the 5' promoter region of the VDR is the FokI SNP (rs2228570, T/C, T = f). The f allele leads to a protein that is 3 amino acids longer and less effective [10] and was associated with a statistically significant increased breast cancer risk in a case-control study [11]. However, other studies did not find any association [12].

In this study, we examined associations of MBC and VDR polymorphisms in a group of retrospectively evaluated 25 MBC patients followed at our clinic with the diagnosis of breast cancer between 1996 and 2009. Various studies have assessed associations between various polymorphisms in the VDR gene and female breast cancer risk but data are lacking in the literature about MBC and VDR gene polymorphisms.

Methods

Study and control groups

The study group consisted of a consecutive series of 27 MBC patients treated at the Dokuz Eylul University Hospital, Izmir, Turkey, between 1996 and 2009. Patient age ranged from 31 to 75 years at the time of diagnosis. Living patients were contacted through their general practitioners. The initial contact was by telephone where possible. Peripheral blood samples (5 ml) were obtained from 4 living MBC patients. Written informed consent was obtained in all cases. Archival paraffin-embedded tissue sections were obtained for 21 deceased patients. Two patients declined to take part in the study.

The control group consisted of 96 healthy males selected from blood donors at the Dokuz Eylul University Hospital, Blood Transfusion service. Informed consent was obtained from all participating patients and controls and the study was carried out after approval of the Ethical Review Committee of Dokuz Eylul University Hospital.

Sample collection and DNA isolation

Peripheral blood samples for all cases and controls were collected into EDTA vials and genomic DNA was extracted from peripheral blood lymphocytes using the standard phenol-chloroform extraction method. Blood was first digested with lysis buffer I (30 mM Tris, 5 mM EDTA and 50 mM NaCl) and lysis buffer II (20% dodecyl sulfate/SDS, 100 mg/ml proteinase K) followed by the extraction with Tris saturated phenol and chloroform-isoamyl alcohol (24:1) and finally recovered by ethanol precipitation. DNA extraction from wax-embedded tissue was from 10 mm sections incubated at 55°C with a lysis buffer and proteinase K followed by the extraction with Tris saturated phenol and chloroform-isoamyl alcohol (24:1) and finally recovered by ethanol precipitation. These genomic DNAs were then used for genotyping of ApaI, TaqI and FokI polymorphism in VDR gene.

Genotype analysis

Genomic DNA was amplified using polymerase chain reaction (PCR). Amplified fragments were digested with restriction enzymes (purchased from MBI Fermentas Inc, Hanover and Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. The ApaI (rs7975232) and TaqI (rs731236) polymorphisms were examined using the primers ApaI/TaqI-forward (5'-AGT AAG AGT CTG GCA AAG ATA GC-3') and ApaI/TaqI-reverse (5'-AAA CAC TTC GAG CAC AAG G-3'). Digestion with ApaI revealed 3 fragments of 104, 216, and 290 bp when the allele "C" was present. Digestion with TaqI resulted in 2 fragments of 400 and 210 bp in the presence of the "C". FokI (rs10735810) was examined with FokI-forward (5'AGC TGG CCC TGG CAC TGA CTC TGC TCT-3') and FokI-reverse (5'ATG GAA ACA CCT TGC TTC TTC TCC CTC-3'). The resulting 267-bp fragment was digested with FokI, producing 2 fragments of 60 and 207 bp after digestion in the presence of the allele "C". Digestion products were visualized in a 3% agarose gel stained with ethidium bromide.

Statistical analysis

Genotype distribution within the groups of cases and controls was compared with values predicted by Hardy-Weinberg equilibrium using the x^2 test. Hardy-Weinberg equilibrium, based on allele frequencies, was verified with the formula: $p^2+2pq+q^2=1$. The differences of allelic and genotypic frequency between case and control groups were also estimated by the Pearson x^2 test or Fisher's exact probability test. The relationships between genotypes and alleles and AMD risks were determined by obtaining the odds ratios (ORs) and 95% confidence intervals (CI) through a logistic regression

method. We used SPSS version 15.0 (SPSS Inc., Chicago, IL) to run the statistical analysis. x^2 values with a probability (p) value < 0.05 were considered statistically significant. Haplotype frequencies for the 3 loci were estimated for cases and controls, using the validated program PHASE v2.1.1.

Results

A total of 25 cases of MBC (age range 31-73 years, mean 57.5±13.35) and 96 healthy control individuals (age range 29-74 years, mean 46.1±12.73) were evaluated. The tumor was localized in the right breast in 56% of the patients and in the left breast in 44% (Table 1). Fourteen (56%) of the patients had lymph node involvement. In 2 cases (15%) steroid receptor analysis was not performed. Of the 23 cases in which receptor analysis was performed, estrogen receptor (ER) and progesterone receptors (PR) were positive in 65.2% (15 patients) and 73.9.6% (17 patients), respectively (15 patients/65.2% ER+/PR+, 6 patients/26.1% ER−/PR−, 2 patients/8.7% ER−/PR+, 1 patient /4.3% ER+/PR−). Moreover, 84% of the cases had invasive ductal carcinoma (IDC), 8% invasive papillary carcinoma (IPC) and 8% invasive lobular carcinoma (ILC). Stage distribution is described in Table 1. At diagnosis, there were distant metastases in 2 patients (8%): 1 patient with bone metastases, 1 patient with bone and pulmonary metastases and both of them had skin invasion as well.

Table 1. General characteristics of male breast cancer patients

Characteristics	*Patients, n*	*%*
Breast		
Right	14	56
Left	11	44
Histology		
Invasive ductal	21	84
Invasive papillary	2	8
Invasive lobular	2	8
Lymph node status		
Positive	17	68
Negative	8	32
Steroid receptor status†		
Estrogen	15	65.2
Progesterone	17	73.9
Age (years)		
0-49	6	24
50-59	6	24
60-69	6	24
70+6	7	28
Stage*		
I	2	8
IIA	2	8
IIB	6	24
IIIA	5	20
IIIB	4	16
IIIC	3	12
IV	3	12
Tumor size (cm)		
< 2	5	20
2-5	17	68
≥5	3	12

*The American Joint Committee Clinical Staging System 2002 was used
†23 cases with steroid receptor analysis

The genotype and allele frequencies of ApaI, TaqI and FokI genotypes and allele frequencies in cases and controls are illustrated in Tables 2, 3 and 4. For each locus, the observed genotype counts were compared to those expected for cases and controls under Hardy-Weinberg equilibrium. To determine whether there was a population substructure in the MBC and control groups, we evaluated Hardy-Weinberg equilibrium for the ApaI (rs7975232), TaqI (rs731236) and FokI (rs10735810) polymorphisms. We found that ApaI (rs7975232) genotype frequencies were in Hardy-Weinberg equilibrium in MBC group (p=0.81) and in the control group (p=0.22) (Table 2). The genotype frequencies of TaqI (rs731236) were in Hardy-Weinberg equilibrium in the control group (p=0.46) and in MBC group (p=0.47) (Table 3). The genotype frequencies of FokI (rs10735810) were in Hardy-Weinberg equilibrium in the control group (p=0.93) and skewed in MBC group (p=0.04) (Table 4).

We did not observe a significant association be-

Table 2. Hardy-Weinberg equilibrium (HWE) for ApaI (rs7975232) polymorphism

ApaI	*Allele*	*Frequency*	*Genotype*	*N*	*Frequency*		X^2	*p-value for HWE*
					Observed	*Expected*		
Controls	A	0.748	AA	51	0.531	0.555		
	C	0.252	AC	41	0.427	0.427	1.46	0.22
			CC	4	0.042	0.065		
Cases	A	0.700	AA	12	0.480	0.492		
	C	0.300	AC	11	0.440	0.420	0.05	0.81
			CC	2	0.080	0.092		

A: adenine, C: cytosine

Table 3. Hardy-Weinberg equilibrium (HWE) for TaqI (rs731236) polymorphism

TaqI	*Allele*	*Frequency*	*Genotype*	*N*	*Frequency*		X^2	*p-value for HWE*
					Observed	*Expected*		
Controls	T	0.766	TT	55	0.573	0.586		
	C	0.234	TC	37	0.385	0.359	0.52	0.46
			CC	4	0.042	0.055		
Cases	T	0.740	TT	13	0.520	0.548		
	C	0.260	TC	11	0.440	0.384	0.51	0.47
			CC	1	0.040	0.068		

T: thymine, C: cytosine

Table 4. Hardy-Weinberg equilibrium (HWE) for FokI (rs2228570) polymorphism

FokI	*Allele*	*Frequency*	*Genotype*	*N*	*Frequency*		X^2	*p-value for HWE*
					Observed	*Expected*		
Controls	T	0.708	TT	48	0.500	0.502		
	C	0.292	TC	40	0.416	0.414	0.006	0.93
			CC	8	0.084	0.085		
Cases	T	0.620	TT	12	0.480	0.384		
	C	0.380	TC	7	0.280	0.472	4.12	0.04
			CC	6	0.240	0.144		

T: thymine, C: cytosine

tween ApaI, TaqI and FokI genotypes of any of the 3 analysed polymorphisms and risk for MBC (Table 5).

The PHASE program resulted in reconstruction of 8 different haplotypes in cases and controls. Overall, the ATT haplotype was the most common haplotype among both groups, comprising 38% among cases and 41.6% in controls, while ACT haplotype was observed in a low frequency among cases (4.0%) compared to controls (10.8%) (Table 6).

Representing genotype (diplotype) distribution based on best pair reconstruction suggested that ACTCTT was over-represented in the cancer group (24.0%). Replacement of TT genotype by CT has drastically brought down the risk with ACTTTT genotype (4.0%) (Table 7).

Discussion

To our knowledge, this is the first study to look for possible modifying effects on MBC risk among VDR polymorphisms. In females breast cancer is seen more frequently in postmenopausal women with a mean age of 63 years, while in men is seen even later at a mean age of 68 years [1,13,14]. The mean patient age in our study was 57.5 years, lower than in other countries.

Table 5. Genotype and allele distribution of ApaI, TaqI and FokI genotypes

Genotypes	*Controls (n=96)*	*Cases (n=25)*	*p-value*	*OR (95% CI)*
ApaI			0.70	
AA	51 (53.1)	12 (48.0)		1[a]
AC	41 (42.7)	11 (44.0)		1.14 (0.456-2.849)
CC	4 (4.1)	2 (8.0)		2.13 (0.348-12.986)
TaqI			0.88	
TT	55 (57.2)	13 (52.0)		1[a]
TC	37 (38.5)	11 (44.0)		1.26 (0.509-3.108)
CC	4 (4.1)	1 (4.0)		1.06 (0.109-10.270)
FokI			0.075	
TT	48 (50.0)	12 (48.0)		1[a]
TC	40 (41.6)	7 (28.0)		0.70 (0.252-1.946)
CC	8 (8.3)	6 (24.0)		3.00 (0.874-10.296)

OR: odds ratio, 95% CI: 95% confidence interval, A: adenine, C: cytosine, T: thymine
[a]Reference genotype/allele

Table 6. Haplotype counts in breast cancer cases and controls (n= 121)

	N	*ATT* *n (%)*	*ATC* *n (%)*	*ACT* *n (%)*	*ACC* *n (%)*	*CTT* *n (%)*	*CTC* *n (%)*	*CCT* *n (%)*	*CCC* *n (%)*
Controls	192	80 (41.6)	38 (19.7)	21 (10.8)	5 (2.5)	22 (11.3)	7 (3.5)	14 (8.2)	5 (2.5)
Cases	50	19 (38.0)	9 (18.0)	2 (4.0)	4 (8.0)	4 (8.0)	6 (12.0)	6 (12.0)	–

Table 7. Odds ratios and 95% confidence intervals for MBC risk associated with diplotype distribution frequencies

Genotype[a]	*Case* *n (%)*	*Controls* *n (%)*	*OR*	*95% CI*
AATTTT	3 (12.0)	18 (18.8)	1[b]	
ACTTTT	1 (4.0)	11 (11.5)	0.55	0.05-5.92
AATCTT	1 (4.0)	5 (5.2)	1.20	0.10-14.19
ACTCTT	6 (24.0)	9 (9.4)	4.00	0.81-19.82
AATTTC	3 (12.0)	11 (11.5)	1.64	0.28-9.58
ACTTTC	2 (8.0)	8 (8.3)	1.50	0.21-10.79
AATCTC	1 (4.0)	9 (9.4)	0.67	0.06-7.35
ACTCTC	1 (4.0)	10 (10.4)	0.60	0.05-6.56
AATTCC	2 (8.0)	5 (5.2)	2.40	0.31-18.55
ACTCCC	1 (4.0)	1 (1.0)	6.00	0.29-124.10

OR: odds ratio, 95% CI: 95% confidence interval
[a]ACTTCC, AACCCC, CCTTCC, CCTTTT= diplotypes found in cases only. CCTCTT, AACCTT, ACCCTT, CCTTTC, CCTCTC, AATCCC= diplotypes found in controls only. [b]Reference genotype/allele

When the histopathologic findings between women and men are compared, several differences emerge due to the fact that classic lobular structure does not occur in men. As male breast does not have lobular elements, the most frequently encountered MBC type is IDC (85-90%) [15]. The results in our study were similar, with a ratio of 84% for IDC and this was significantly higher than the other histological types; no patient had ductal carcinoma *in situ* [16,17].

ER positivity is more frequent in MBC compared to women. In different studies, ER and PR positivity ranged from 75 to 93% in males [15,18]. In our study, steroid receptor analysis was performed in 23 patients and showed that PR positivity was similar to the literature, but the ER positivity was slightly lower.

Carcinoma of the male breast has many similarities to breast cancer in women, but the diseases have different genetic and pathologic features. Both *BRCA1* and *BRCA2* mutations can cause breast cancer in women, but only *BRCA2* mutations confer a significant risk to men [19,20]. Several studies have shown evidence for the putative role of VDR polymorphism in the risk of breast cancer in females [12,21,22]. In contrast, several prior studies did not report an increased risk for breast cancer in females associated with VDR polymorphisms [23,24]. To our knowledge, our study is the first to investigate the putative role of VDR polymorphism in the risk of MBC.

Among the 3 polymorphisms examined, the linkage disequilibrium between ApaI and TaqI was near to strong linkage disequilibrium. This is consistent with what has previously been reported [8]. In the present study, the ATT haplotype was most common among Turkish population whereas ACT haplotype (4.0%) was lower in cancer cases.

There are difficulties, however, in equating different polymorphisms between studies. For example, the strength of linkage disequilibrium can vary significantly between populations [25], resulting in misclassification of the "at-risk" locus. VDR allele frequencies may also vary within Caucasian populations: of 10 polymorphic genes assessed in a Finnish study, the prevalence of the 3' TaqI polymorphism was significantly different from other Caucasian populations [26]. That the Caucasian populations are heterogeneous with respect to the VDR gene is one possible explanation for such discrepancies between populations-based VDR association studies.

As true with any research study, the present study also has its own strengths and limitations. In the demographic analysis limited factors were considered and for the majority of them the analysis was unmatched, since information could be achieved only from surgical specimens. Other likely limitation was the small sample size. But even with these limitations, the present study makes a substantial endeavor in enriching our knowledge towards better understanding of vitamin D receptor gene polymorphism(s) and MBC risk in Turkish population, and this issue can be addressed more reli-

ably by recruiting a greater sample size to confirm these results. Moreover, to our knowledge, the present study is the first report over a possible role of VDR polymorphisms and MBC risk.

In conclusion, we didn't find any evidence that differences in the oncogenic properties of the VDR gene Apal, Tacl and Focl alleles could confer a genetic predisposition to MBC carcinogenesis. Additional studies are needed to elucidate the role of genetic variations in VDR and MBC risk.

Acknowledgement

This study was supported by the The Scientific and Technological Research Council of Turkey (TUBITAK) research fund with code number 108S251 and was presented in the form of poster at the 11th National Congress on Medical Biology and Genetics, 28-31 October 2009 in Bodrum, Turkey.

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