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Association of Vitamin D Receptor-FOKI Gene Polymorphism with Breast Cancer Risk in Iraqi Female Patients

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Abstract: ***Background: Breast cancer is the most frequent cancer among women worldwide. In Iraq, it is accounting about one third of the registered women cancers. Different studies have shown that the gene polymorphism in vitamin D receptor (VDR-FOKI) was associated with the susceptibility and pathogenesis of breast cancer. Objective: The study aimed to examine the genotyping utility of Vitamin D receptor (VDR-FokI) gene polymorphism as predictor for the occurrence and progression of breast cancer in Iraqi women. Materials and Methods: This case control study was comprised of two groups, the first group consisted of 300 women histopathologically diagnosed with breast cancer either familial or sporadic with age 22-77 year, the second group included 200 healthy women with age 22-78 year, DNA was extracted from blood and genotyping analysis was carried out by PCR-RFLP. Results: The genotypic analysis revealed that allele frequency of TT genotype (29%), TC genotype (48%), and CC genotype (23%) in breast cancer group, while 64.5%, 30.5% and 5% in the control group respectively. The risk of breast cancer was significantly increased with VDR-FOKI SNP by about three and five folds in heterozygous and homozygous of mutant allele genotypes in patients (OR=3.25, 95% C. I= 2.16-4.89, P=0.0001 and OR= 5.00, 95% C. I= 2.81-8.92, P= 0.0001 respectively) when compared with the homozygous of normal alleles genotype after adjustment for age and BMI. The C allele frequency increased the risk of disease by three and half times when compared with those of T allele (OR= 3.49, 95% C. I= 2.6-4.67, P < 0.0001). Conclusion: We conclude from this study that the gene polymorphism of VDR-FOKI was associated with high risk of development and progression of breast cancer.***

Keywords: breast cancer, VDR-FOKI, gene, polymorphism, females, Iraq

1. Introduction

Breast cancer is the most common cancer among women, comprising 23% of female cancers (Parkin, 2012). According to Globocan, annually 1.38 million new women diagnosed with breast malignancy worldwide, and the disease mortality rates were 458,367; of these about 60% of the death were recorded in less developing regions of the world (Ferlay et al., 2012).

In Iraq, breast cancer is the most common type of female risk, representing 33% of the enrolled female breast cancer patients as Iraqi Cancer Registry (Iraqi Cancer Board ICB, 2014). Breast cancer is a heterogeneous disease regarding to its morphology, invasive behavior, metastatic capacity, hormone receptor expression and medical effect. There are numerous risk factors for breast cancer including genetic factors which account for 25-30% of the incidence, from this percentage only 15-30% genetic component of breast cancer is due to known familial genes such as BRCA1 and BRCA2 and the others are sporadic (www.broad.mit.edu/mpg/haploview; Mohr et al; 2012).

Single nucleotide polymorphism (SNP) is one of the genetic markers that commonly used in the last years to study genetic phenotypes (www.broad.mit.edu/mpg/haploview; Mohr et al; 2012; AnneMarie et al., 2015). SNPs are used in routine works for prediction of the basis of tumor characteristics. Numerous studies have found that vitamin D reduced the cells production and enhanced cells differentiation in breast cancer cell lines and tumor samples, hence plays a protective role against breast cancer development (Buttigliero et al., 2011; Cheung et al., 2012; Neuhouser et al., 2010). The active form of vitamin D linked with vitamin D receptor (VDR) and the ligand/receptor compound manages translation of the genes implicated in cell cycle, apoptosis and differentiation.

VDR gene located on chromosome 12q13-14 has multiple polymorphisms, VDR-FOKI (rs 2228570) gene polymorphism (Met 1 Thr) was the most essential SNP in starting codon. Alterations in VDR expression and action could prompt deregulation of vitamin D uptake (Sinnett et al., 2009).

A number of studies have established a reduction in VDR expression in breast cancer cells compared with normal breast cells, decreased function and activity of VDR in breast cancer cells might be due to VDR gene polymorphism. Practical study has recognized that the VDR-FokI polymorphism prompts a shorter VDR protein by changing a translation initiation site (Alimirah et al., 2011).

This study aimed to examine the genotyping utility of VDR-FokI gene polymorphism as a predictor for occurrence of breast cancer in Iraqi women and to evaluate the benefits of investigated gene polymorphism in the prediction of the progression of disease.

2. Material and methods

This case-control study included the consent protocol approved by medical ethics committee Faculty of Medicine/Kufa University. Written information approval

was obtained from all participants. It was conducted during the period from April 2014 to February 2019. The work was carried out in the biochemistry laboratory in biochemistry department and molecular biology laboratory in the anatomy and histology department/ College of Medicine/University of Kufa.

A total of 200 healthy individuals with age of 22-78 years compared with 300 females histopathelogically diagnosed with breast cancer either familial (16.3%) or sporadic (83.7%) with age 22-77 years, they were collected from the oncology unit in AL-Sadder medical City teaching hospital in Al-Najaf Province, they were from middle and south regions of Iraq. Any subject suffered from other types of malignancies or chronic diseases were excluded from this study.

DNA extraction and gene analysis:

DNA was extracted from blood by using (ReliaPrep™ promega extraction kit). VDR FokI genotype was analyzed using PCR- RFLP. The DNA was amplified by using specific primers (Harris et al.,1997),F:5′-AGCTGGCCCTGGCACTGACTCTGCTCT-3′ and R:5′-TGGAAACACCTTGCTTCTTCTCCCTC-3′.The amplification was accomplished with a 25 µl reaction mixture containing of 10-100 ng DNA, 15 pmol of each primer, master mix contains of (2.5 µl 10 mM dNTPs, 1.5 µl of 20 mM MgCl2, 0.3 µl of 5 U/ µl Taq polymerase with 2.5 µl of 10X Taq Buffer) (Promega, USA). PCR conditions were: Initial denaturation at 94°C for 6 minutes followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds, extension at 72°C for 45 seconds and final extension at 72°C for 5 minutes. The amplicons were digested with 10 units of FokI enzyme (New England Biolabs, USA) by incubating at 37°C for 1 hour and visualized on 2.0 % agarose gel stained with ethidium bromide.

Statistical analyses

Statistical analyses were performed using the SPSS windows software (SPSS Inc., Chicago, IL) Data were expressed as mean ±SD by using t- test. Genotype and allele frequencies in patients and control group were tested by multinomial logistic regression analysis. Differences in clinical characteristics of breast cancer patients and VDR-FOKI gene polymorphism in recessive model tested by binary logistic regression, values of P< 0.05 were considered statistically significant. Hardy–Weinberg equilibrium (HWE) was calculated using the online software web-Assotest (www.ekstoem.com). Genetic power was measured using the online software OSSE (osse.bii.a-star.edu.sg).

3. Results

The demographic characteristics of patients and control groups

The distribution of demographic characteristics for patients and control are shown in table (1). There were significant difference between two groups in Educational level and Parity (P<0.05).

Table 1: Demographic characteristics of patients and controls

Characteristics	Patients no.= 300	%	Control no.= 200	%	P-value
Marital status					
Married	196	65.40%	125	62.50%	0.07
Unmarried	28	9.30%	10	5%	
Widow/Divorced	76	25.30%	65	32.50%	
Residence					
Urban	138	46%	90	45%	0.82
Rural	162	54%	110	55%	
Educational level					
Low	195	65%	144	72%	0.016
Middle	75	25%	36	18%	
High	30	10%	20	10%	
Parity					
Nullparous	26	8.70%	14	7%	0.03
1	53	17.70%	27	13.50%	
2 – 3	85	28.30%	40	20%	
≥ 4	136	45.30%	119	59.50%	

P< 0.05: significant.

The clinicopathological parameters in patients with breast cancer.

The clinicopathological criteria of breast cancer patients are shown in table (2).

Table 2: Clinicopathological parameters in 300 breast cancer patients

Parameters	No. of patients	%
First degree relative with breast cancer		
Positive	49	16.30%
Negative	251	83.70%
Laterality		
Rt. Breast	175	58.30%
Lt. Breast	90	30%
Both	35	11.70%
Mass	109	36.30%
Mastectomy	191	63.70%
Histopathological type of Breast Cancer		
IDC	234	78%
ILC	45	15%
Others	21	7%
Histopathological information:-		
Stage of Tumor		
Stage 0 (Tis)	19	6.30%
Stage I	29	9.70%
Stage II	109	36.30%
Stage III	120	40%
Stage IV	23	7.70%
Grade of Tumor		
I (well)	48	16%
II (moderately)	148	49.30%
III (poorly)	104	34.70%
IHC information:-		
ER- status		
Positive	206	68.70%
Negative	94	31.30%
PR- status		
Positive	209	69.70%
Negative	91	30.30%

IHC: immunohistochemistry; ER: estrogen receptor; PR: progesterone receptor; T: size of tumor; N: lymph node involvement;

M: metastasis; IDL: invasive ductal carcinoma; ILC: invasive lobular carcinoma; Rt: right; Lt: left.

Genotyping analysis of patients and healthy control individuals

The current study investigated the relationship between gene polymorphism of VDR-FOKI (rs 2228570) and risk of breast cancer in a 500 age matched patients and healthy individuals, The digestion of the PCR-product by a restriction enzyme FOKI explored three patterns: One band (273 bp) for homozygous normal alleles, three bands (273, 198 and 75 bp) for heterozygous genotype, and two bands (198 and 75 bp) for homozygous mutant alleles figure (1).

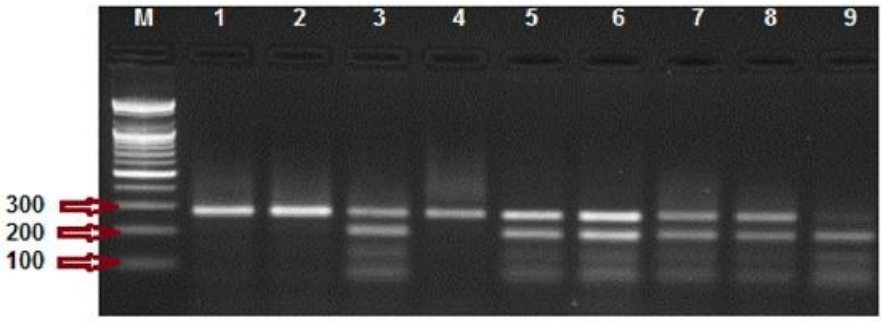

Figure 1: Genotyping result of VDR-FOKI gene. Lane M: DNA Ladder. Lane 1, 2 and 4 are normal alleles genotype TT(273 bp), Lane 3, 5, 6, 7 and 8 are heterozygous genotype TC(273, 198, 75) and Lane 9 homozygous mutant alleles genotype CC(198, 75).

The genotypes and allele frequencies of VDR-FOKI gene polymorphism in patients and control groups

The genetic power was calculated for VDR-FOKI gene (T/C), It is equal to (100 %), most these findings revealed the compatibleness of sample size with study design and also high relation between VDR-FOKI gene polymorphism and breast cancer disease. Genotyping frequencies of VDR-FOKI gene were consistent with Hardy Weinberg Equilibrium in both breast cancer group (P= 0.53) and control group (P= 0.43).

The multinomial logistic regression analysis of results indicated that the VDR-FOKI genotype frequencies of TT, TC and CC were 29 %, 48 % and 23 % in breast cancer group and 64.5%, 30.5% and 5% in control group respectively. The heterozygous was increased the risk of disease by three folds with respect to those of the normal alleles (OR=3.25, 95% C. I=2.16-4.89, P= 0.0001). On the other hand, the homozygous mutant alleles was increased the risk of disease by five folds higher than that found in normal alleles (OR=5.00, 95% C. I=2.81-8.92, P= 0.0001) after adjustment for age and BMI. The risk of genotype regarding to dominant inheritance model (TC+CC) for patients was about four times higher than TT genotype (OR=4.4, 95% C. I= 3.02-6.48, P=0.0001).

The frequency of T allele equal to 53 % and 79.75 % in breast cancer and control groups respectively, while the frequency of C allele equal to 47% in breast cancer which is higher than C allele frequency in control group 20.25 % (P< 0.0001), the C allele frequency increased the risk of breast cancer by three and half times when compared with those of T allele (OR=3.49, 95% C.I= 2.6-4.67, P < 0.0001) as shown in table (3).

Table 3: Genotype frequencies of VDR-FOKI gene polymorphism in patients and control and their associations with the risk of breast cancer

Genotype of VDR-FOKI (T/C)	Patients No.	%	Control No.	%	Adjusted OR	95% C.I	P-value
TT	87	29	129	64.5	1 (Ref.)		
TC	144	48	61	30.5	3.25	2.16-4.89	0.0001
CC	69	23	10	5	5.00	2.81-8.92	0.0001
TC + TT vs CC	213	71	71	35.5	4.4	3.02-6.48	0.0001
T Allele	318	53	319	79.75	Ref.		
C Allele	282	47	81	20.25	3.49	2.6-4.67	< 0.0001

Multinomial logistic regression stratified adjusted for age and BMI; No: number; C.I, confidence interval; OR, odds ratio, Ref, Reference.

The relation between clinical characteristics of breast cancer patients and VDR-FOKI gene polymorphism in recessive model

The breast cancer patients stratified to those with and without family history, the results of binary logistic regression revealed a higher risk of breast cancer in patients without family history associated with univariate genotype (CC) alleles than those of multivariate recessive inheritance model (TT+TC) alleles, VDR-FOKI was statistically significant increased by twice folds among women without family history than those who have first degree relatives of disease (OR=2.28, 95% C.I=1.18-4.39, P=0.014). Other

factors modifying the risk of breast cancer such as age, stage, grade, estrogen receptor and progesterone receptor status were estimated, the results showed a highly significant association between VDR-FOKI genotype univariate (CC) alleles and those of multivariate (TT+TC) alleles in higher grade (III) of breast cancer by four times more than those of lower grade (I and II), (OR=3.98, 95% C. I=2.28-6.9, P<0.0001). Estrogen receptor status and progesterone receptor status exhibited a moderate risk factor in patients who carrying univariate (CC) alleles than those of multivariate (TT+TC) alleles by approximately one and half in those with positive estrogen receptor and progesterone receptor than those with negative receptors (OR=1.53, 95% C. I= 0.68-2.9, P=0.042) for estrogen receptor and (OR=1.62, 95% C. I= 0.82-3.15, P=0.016) for progesterone receptor. However, no significant association were recorded with regard to patients age and stages of disease as appeared in table (4).

Table 4: The relation between clinical characteristics of breast cancer patients and VDR-FOKI gene polymorphism (recessive model)

Variable	n=300	Distribution of genotype in cases		OR[a]	(95% C.I)	P-value
		TT+TC (231) (R%, C%)	CC (69) (R%, C%)[c]			
1st degree Family history:						
No Family history	251	200 (79.7, 86.6)	51(20.3, 73.9)	2.28	(1.18-4.39)	0.014
Having Family history	49	31 (63.3, 13.4)	18 (36.7, 26.1)			
Age (year)						
>50	140	113 (80.7, 48.9)	27 (19.3, 39.1)			
≤50	160	118 (73.7, 51)	42 (26.3, 60.1)	1.4	(0.8-2.6)	0.15
Stage of disease:						
Initial stages/0+I+II	157	120 (76.4, 51.9)	37 (23.6, 53.6)			
Advance stages/III + IV	143	111 (77.6, 48.1)	32 (22.4, 46.4)	0.96	(0.58-1.7)	0.145
Grade:						
Lower/ I + II	196	168 (85.7, 72.7)	28 (14.3, 40.6)			
Higher/ III	104	63 (60.6, 27.3)	41 (38.5 , 58)	3.98	(2.28-6.9)	<0.0001
ER status:						
ER - negative	94	74 (78.7, 32)	20 (21.3, 28.9)			
ER – positive	206	157 (76.2, 67.9)	49 (23.8, 71)	1.53	(0.68-2.9)	0.042
PR status:						
PR - negative	91	70 (76.9, 30.3)	21 (23.1, 30.4)			
PR – positive	209	161 (77, 69.7)	48 (23, 96.6)	1.62	(0.82-3.15)	0.016

[a]OR Adjusted odds ratio, [c] R(Row), C(Column), C.I, confidence interval, P<0.05: statistically significant.

4. Discussion

Genotypes and allele frequencies of VDR-FOKI gene polymorphism

This study appeared increase risk of cancer in females who carried out heterozygous and homozygous mutant alleles when compared with patients who carried out homozygous of normal alleles. Furthermore, there was a higher risk of disease associated with C allele genotype. Results of this study were agreement with the results by genotype reports in different populations in USA (Haidan et al., 2017; Katherine, 2013). Also, meta-analysis was established a higher association between CC genotype and risk of breast cancer in European females (Tang et al., 2009), in Afro-American and Hispanic females was identified that increased association of VDR- FOKI (CC) in patients with breast cancer (Dhruva et al., 2013). In non Hispanic and Hispanic white females found a strong association between risk of breast cancer in one allele or two alleles genotypes polymorphism (Raimondi et al., 2009; Rollison et al.,2012).

The data of present study were also consistent with many studies that comparing allele frequency of VDR- FOKI in breast cancer individuals and found strong association with C allele frequency in female breast cancer as reported in Denmark (James et al., 2005), in Germany (Swami et al., 2006), and in Japan (Takeyama et al., 2007). On the other hand, the present study disagrees with other reports which found that there were no relationships in VDR- FOKI and breast cancer as established in Iranian women (Shahbazi et al.,2013), in Chinese Caucasian women (Baohong et al., 2014), and in Caucasian women of UK (Curran et al.,2006).

The relation between breast cancer patients characteristics and VDR- FOKI gene polymorphism

In regard to the relevance of VDR- FOKI gene polymorphism with family history of disease, stage, grade of disease, and ER, PR status. The results showed that the occurrence of breast cancer increased in females without first degree family history of disease by about twice times higher than the others, it is similar to that recorded in French Canadian population (Le Marchand, and Wilkens, 2008), in United Kingdom that found breast cancer patients were associated with no family history at higher risk of breast malignancy when compared with other cases (Michelle et al., 2004).

Moreover, there were four folds increased in the risk of disease in patients associated with higher grade of breast cancer when compared with those of lower grade patients, while there were no significant associations between the polymorphism in VDR- FOKI and tumor stage. The results of current study are in full agreement with Anneza et.al who found C allele was associated with the presence of lymphovascular invasion and poorly differentiated tumors (Anneza et al.,2014), as well as Alimirah et.al who found increased expression of proinflamatory gene that characterized by (CC) variant in breast cancer as the possible clinical marker of tumor aggressiveness (Alimirah et al., 2011). Also, this study is consistent with Mishra et.al

who identified there were no significant associations between the polymorphism in VDR- FOKI and tumor stage (Mishra and Sarkissyan, 2013).

The present study observed significantly increased risk of disease in females with positive estrogen and progesterone receptors malignancies when compared with negative receptos tumors, these results are compatible with Nurses' Health Study in USA that proposed an opposite connection with hormone receptor negative with breast cancer (Katherine, 2013). On the other side, Some studies disagree with these results demonstrated that the independency of hormone receptor level with the defense effect of vitamin D in the risk of breast cancer (Bertone-Johnson et al., 2005). Finally, since C allele leads to VDR less efficient, the relative risk and incidence of breast cancer are elevated with C allele, collectively all these studies indicated that the defect in VDR- FOKI would be better suiting for treatment with vitamin D based on their VDR-FOKI genotypes classification (Li et al., 2007; Rajendra et al., 2013).

We concluded from this study that gene polymorphism of VDR-FOKI was associated with development of breast cancer women with CC genotype have higher risk of developing more progressive breast cancer compared to TC and TT genotypes.

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