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The Potential Association Between Polymorphism in BRCA1-Associated RING Domain (*BARD1*) Gene and Breast Cancer in Jeddah Province

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Abstract: The current study was aimed to identify the potential association between BRCAI-Associated RING Domain (BARD1) gene polymorphism and women breast cancer in Jeddah province, Saudi Arabia. It is a case-control study where blood samples of participants were collected. A total number of 200 subjects from breast cancer patients and control women (100 for each) were examined for demographic characteristics and SNP mutation using Tetra-Primer amplification-refractory mutation system-polymerase chain reaction technique, to identify allele distributions for *BARD1* rs2228456(A/G), rs111367604 (C/A) and rs28997576 (C/G) SNPs that might be associated with the disease progression. The demographic results revealed that 87% of breast cancer patients were <40 years old. Where 64% of all patients have no family history of breast cancer, 26% of them have used contraceptive pills and 38% with previously psychological problems. Furthermore, invasive ductal carcinoma (IDC) was the most common type (73.43%). The Tumor, lymph node and metastasis (TNM) classification results revealed the tumor stage (T1), Lymph node (N0) and Metastasis (M0) were as the highest prevalent stages, 48.40%, 67.20% and 95.30, respectively. The results of tetra ARMS–PCR, revealed heterozygous genotype pattern for both patient and control samples using *BARD1* SNP rs2228456 (A/G), while normal homozygous (non-mutant) genotypes were shown for both patient and control samples using both *BARD1* rs111367604 (C/A) and rs28997576 (C/G) SNPs. In conclusion, no relationship was found between breast cancer and *BARD1* polymorphisms using the chosen SNPs (rs2228456, rs111367604 and rs28997576).

Key words: Breast Cancer · BARD1 · BRCA1 · SNPs · Tetra-ARMS-PCR

INTRODUCTION

Breast cancer represents one of the most communal malignant tumors. It has exceeded lung cancer with the estimation of 2.3 million new cases representing 11.7% of all cancer types. In Saudi Arabia breast cancer represents about 29% of all cancers that affect Saudi women, making it one of the most commonly diagnosed types of cancer. According to the most recent cancer mortality survey, breast cancer was considered the ninth cause of death in Saudi women [1]. Moreover, above 50% of cases with breast cancer are detected at a late stage [2].

Breast cancer genes that have products of known interaction with *BRCA1* or *BRCA2* are frequently involved in DNA repair or cell cycle regulation. They are particularly attractive candidate genes for breast cancer susceptibility [3]. Functionally related genes

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such as *ATM*, *CHEK2* and *BARD1* (normally repair damaged DNA), have been analyzed for the presence of their germline mutations, which may explicate the increased breast and/or ovarian cancer risk [4]. The last one is the primary concern of this research study, BRCA1-associated RING Domain Protein (*BARD1*) located on 2q35 and encodes for 777 amino acids. It's consisted of 11 exons, sharing both structural and functional similarities with BRCA1. In addition, *BARD1* interacts with *BRCA1* in DNA double-strand break (DSB) repair and also in apoptosis initiation [4]. Both proteins possess an amino-terminal RING (really interesting new gene) finger motif and two carboxy-terminal BRCT (BRCA1 C Terminus) domains. The formation of the heterodimer complex between *BARD1* and *BRCA1* is mediated by their RING finger motifs. This binding seems to be critical for several tumor suppressor functions of *BRCA1* as well as to stabilize both proteins [5]. Deleterious *BRCA1* missense mutations such as p.Cys61Gly disturb the interaction between these two proteins [6]. *BARD1* gene codes for four ankyrin repeats that mediate protein–protein interaction and are frequently present in many proteins are involved in transcriptional regulation and checkpoint control [7, 8]. *BARD1* is a nuclear protein and has similar tissue distribution as *BRCA1*; the highest expression rate for both proteins was reported in testis, spleen and actively proliferating cells as well as in cells that undergo apoptosis [9]. It is well established that the expression of both genes is essential for cell survival. Furthermore, *BARD1* accumulates in the cytoplasm as a consequence of the low expression level of *BRCA1*. This accumulation plays an important role in the apoptosis initiation of a cell [10, 11]. Moreover, the absence of *BARD1* is lethal to the cell. Homozygous *Bard1*-null mutation in mice causes early embryonic lethality. The cells isolated from these mice possess genomic instability. Likewise, the phenotype of *Bard1*-null mutation in mice is very close to that of *Brca1*-null mice, highlighting the similar functional effect of both genes disruption [12]. The presence of adequate early diagnostic methods with the best available treatments represents a good chance that breast cancer to be cured. Since as mentioned recently about the circulation of tumor DNA (ctDNA) in the patient's bloodstream, the blood could be drawn from a patient and the ctDNA could be extracted for further molecular analysis. Therefore, the current study was aimed to identify the potential association between BRCAI-Associated RING Domain (*BARD1*) gene polymorphism and women breast cancer in Jeddah, Saudi Arabia using rs2228456(A/G) SNP exon 10, in addition to rs111367604 (C/A) and rs28997576 (C/G) SNPs in exon 11 tetra-primer amplification refractory mutation system–polymerase chain reaction (ARMS-PCR).

MATERIALS ANDMETHODS

Human Subjects: The research study has conducted on 200 women living in Jeddah city aged 18-82 years, 100 for each health and breast cancer patient. Between 2014 and 2016 period of time, participants' blood samples were collected from King Abdulaziz University Hospital and Medical Reference Clinics in Jeddah city. At the same time, the participants filled in details about their age, family history, consumption contraceptive pills and early detection of tumors by mammography screening.

Sample Collection: Peripheral blood samples were collected from women diagnosed with breast cancer and healthy women as control at King Abdulaziz University Hospital and Medical Reference Clinics in Jeddah city. The present study was approved by Biomedical ethics unit at King Abdulaziz University with the No. of (2/36/40591).

Extraction of DNA: Genomic DNA was extracted using the ReliaPrepTM Blood gDNAMiniprep System kits. It was isolated from the whole blood samples that were stored in the EDTA coated Lavender top tube, Franklin Lakes, NJ, USA.

Primer Design: *BARD1* gene sequence was obtained from the GenBank nucleotide sequence database. The tetra-primer ARMS were designed using primer BLAST software (Table 1).

Amplification for *BARD1* Gene: *BARD1* gene has been amplified using the tetra-primer ARMS PCR primers: PCR amplification products were obtained using a final volume of 25µl and prepared as 100 ng genomic DNA, 12.5µl GoTaq® Green Master Mix, 2µl of mix primer and sterile distilled water up to 25µl in a Thermal cycler (master cycle personal, Eppendorf, Germany). Different PCR programs were used for each SNP. For rs2228456 SNP, the program was touch-down; at 95°C for 5 min,

Table 1: Tetra-arm Primer sequences for the three chosen SNPs

SNPs	System	Primer sequence (5' –3')	Allele	Tm°C	Amplicon (bp)
rs2228456 (A/G)	Forward outer primer (F1)	CCTGTAGCTGTTGAAAGGGCAGAAGTT	-	57	538
	Reverse inner primer (R2)	GGGGTAAAAGCATGTCTACGAAGAAAAGTAT	A normal	59	194
	Forward inner primer (F3)	AGGAATTTCATACTTTTCTTCCTGTTCACG	G mutant	58	404
	Reverse outer primer (R4)	GGGTAATTGCTATGAGTGGAAATGTAGAC	-	59	-
rs111367604 (C/A)	Forward outer primer(F1)	CATAGATGATATACTGTGTGCAGAAGCG	-	58	465
	Reverse inner primer (R2)	CATCCAAAGGACAACCTTATTAAGCTCG	C normal	58	161
	Forward inner primer (F3)	CTGGCCCCCACCTGCAGTGAA	A mutant	60	330
	Reverse outer primer (R4)	GGCTATGTGCATAAGGTGTACAAGAAAC	-	58	-
rs28997576 (C/G)	Forward outer primer(F1)	GATGATATACTGTGTGCAGAAGCGCTG	-	60	413
	Reverse inner primer (R2)	CCATCCAAAGGACAACCTTATTAAGCTCG	C normal	60	160
	Forward inner primer (F3)	TCTGGCCCCCACCTGCAGTGAG	G mutant	63	303
	Reverse outer primer (R4)	CTCCTCCAAATGTTTCAGGTAAGGG	-	58	-

15 cycles at 95°C for 45s, 66°C for 45s and 1 min at 72°C and 20 cycles at 95°C for 45s, 72°C for 1 min and 45s at 60°C and 72°C for 5 min. For SNP s111367604, the program was as follows; at 95°C for 5 min and 35 cycles at 65°C for 30s and 72°C for 5 minutes. For SNP rs28997576, the program was as follows; at 95°C for 5 min and 35 cycles at 58°C for 30s and 72°C for 5 minutes. Electrophoreses for PCR products were done using 2% agarose gel that stained with 3 µl Ethidium Bromide (EtBr). Bands on the gel were visualized in the gel documentation system.

Statistical Analysis: Package for Social Sciences (SPSS version 20) from SPSS Inc., Chicago, IL, U.S.A was used to statistically analyze the current data. Data were presented as mean +/- standard deviation or numbers as appropriate. The continuous variables between two groups were made using unpaired sample "t" test more than two groups using One Way ANOVA (LSD) test and between categorized data using Chi-Square test. A probability (P).

RESULTS

Table 2 represents the demographic characteristics of participants. In this study, the mean age of patients was relatively higher than control (53.81±11.74 and 33.64±12.17, respectively). The patient's classification of age revealed that 87% of them were > 40 years old and 13% of them were ≤ 40 years old. Positive family history percentages were found to be higher in patients compared to control (36% and 26%, respectively). Compared with control, the administration of contraceptive pills was higher among patients (26%) than control (19%). Psychological problems affected patients represent 38%. The periodic screening percentages were higher in patients compared to control (13% and 36%, respectively).

For patients, positive family history, administration of contraceptive pills, positive psychological problem and periodic screening percentages were higher in those > 40 years old patients compared to ≤ 40 years old.

The classifications of tumors based on their sides were represented in Table 3, which revealed that the tumor prevalence was higher in the left side (45%) compared to the right side (42%), then both sides (13%) with a significant difference between them (P = 0.001). The Positive and negative expression of ER, PR and HER2 receptors positive expression were (90%, 73% and 28%) and the negative expression was (10%, 24% and 67%) respectively.

Stage-based, Type-based and the TNM staging-based classification results of the tumors in all patients are represented in Table 4. It was found that the most frequent stages of the tumor were stage I (54.68%), followed by stage III (20.31%), then stage II (18.75%) and finally stage 0 (6.25%) with significant differences (P = 0.001).

In addition, the most common tumor type was realized as IDC (73.43%), followed by other types (12.5%), then type DCIS (10.93%) and finally type ILC (3.12%) with significant differences between one side (right or left) and both sides patients (P= 0.001) as shown in Table 4.

The tumor "T" stage percentages were as follows: T1 (48.40%), T2 (31.20%), T3 (14.10%) and T4 (6.20%), with significant differences among them (P= 0.001). Lymph node "N" percentages were N0 (67.20%), N1 (28.10%) and N2 (4.70%), with significant differences among them (P= 0.001). Meanwhile, metastasis "M" percentages were M0 (95.30%) and M1 (4.70%) with a significant difference between them (P= 0.001) as presented in Table 4.

Table 2: Demographic characteristics of all participants

Data	Control (n=100)	Patients(n=100)	Aging>40 yearsold patients	Aging ≤ 40 yearsold patients
Age	33.64±12.17 (18.00-63.00)	53.81±11.74 (28.00-82.00)	(n= 87) 87%	(n=13) 13%
Positive familyhistoryofcancer	26 (26%)	36 (36%)	33 (33%)	3 (3%)
ContraceptivePills	19 (19%)	26 (26%)	24 (24%)	2 (2%)
Psychological Problems	-	38 (38%)	35 (35%)	3 (3%)
Periodic screening	5 (5%)	13 (13%)	13 (13%)	ÜÜ

Table 3: Percentage of the side for the tumors and description of the receptors of all patients

Data	Patients (n=100)	*P*-value
Side		
Right	(42%)	
Left	(45%)	P= 0.001
Both Sides	(13%)	
Receptors		
ER		
Positive	(90%)	
Negative	(10%)	P = 0.001
PR		
Positive	(73%)	
Negative	(24%)	P = 0.001
HER2		
Positive	(28%)	
Negative	(67%)	P = 0.003

Table 4: Percentage of tumor stages, types and (TNM) classification percentage for patients

Data	Patients (n=64)	*P*-value
Stage		
0	(6.25%)	
I	(54.68%)	P = 0.001
II	(18.75%)	
III	(20.31%)	
Type		
Invasive ductal carcinoma	(73.43%)	
Ductal carcinoma in situ	(10.93%)	P = 0.001
Invasive lobular carcinoma	(3.12%)	
Others	(12.5%)	
(TNM)		
Tumor		
T1	(48.43%)	P = 0.001
T2	(31.25%)	
T3	(14.06%)	
T4	(6.25%)	
N -Lymph node		
N0	(67.18%)	P = 0.001
N1	(28.12%)	
N2	(4.68%)	
Metastasis		
M0	(95.31%)	P = 0.001
M1	(4.68%)	

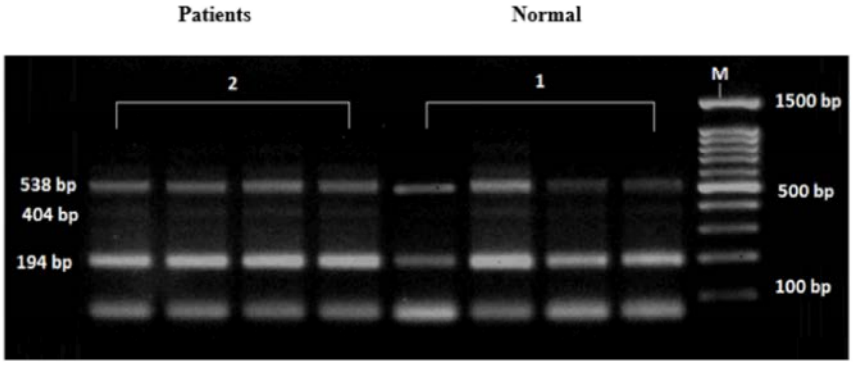

Fig. 1: Agarose gel electrophoresis showing the genotype results for rs2228456(A/G) SNP of *BARD1* gene, both control and patients' samples have a heterozygous A/G genotype that produces three bands sized as538 bp, 404 bp and194 bp, M refers to 100 bp ladder

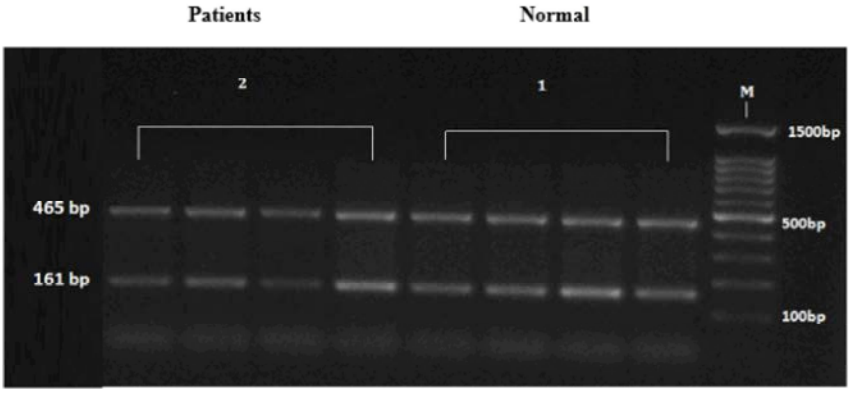

Fig. 2: Agarose gel electrophoresis showing the genotype results for rs111367604 (C/A) SNP of *BARD1* gene, both control and patients samples have a homozygous A/A normal genotype that produce double band sized as 465 bpand161 bp, M refers to 100 bp ladder

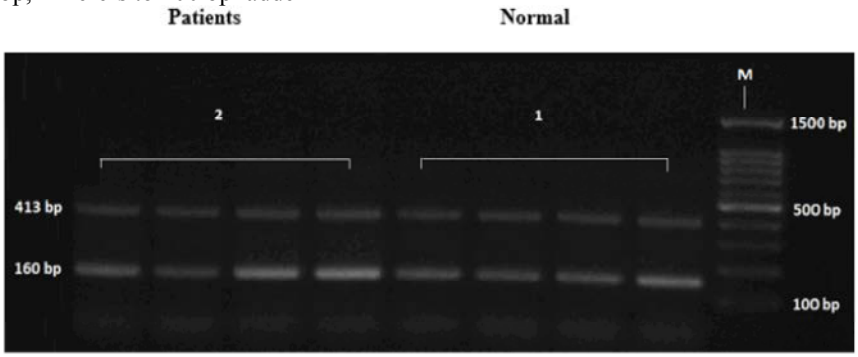

Fig. 3: Agarose gel electrophoresis showing the genotype results for rs28997576(C/G)SNP of *BARD1* gene, both control and patients samples have a homozygous C/C normal genotype that produce double band sized as413 bpand160 bp, M refers to 100 bp ladder

Genotypes for *BARD1* Gene: Genotypes for *BARD1* gene rs2228456, rs111367604, rs28997576 SNPs results are shown in Figures 1, 2 and 3. The genotyping results for *BARD1* rs2228456 (A/G) SNP, which transforms the amino acid Cys to Arg in the produced protein. Three bands with molecular sizes of 538 bp, 404 bp and 194 bp representing heterozygous genotypes for BARD1 rs2228456 (A/G) SNP in both control and patient's groups were observed (Fig. 1).

The genotyping results of *BARD1* rs111367604 (C/A) SNP, which transforms the amino acid Val to Phe in the produced protein revealed double bands with the molecular sizes of 465 bp and 161 bp representing the existence of homozygous genotypes (C/C) for the normal allele in both control and breast cancer patients (Fig. 2). The genotyping results of *BARD1* gene rs28997576 (C/G) SNP which transforms the amino acid Val to Leu in the produced protein were shown in Fig. 3. Double bands have also appeared with the molecular size of 413 bp and 160 bp showing the presence of homozygous genotypes (C/C) for the normal allele in all control and breast cancer patients (Figure 3).

DISCUSSION

Breast cancer has the main influence on women's health worldwide. It is considered the most common malignant tumor and it comes after lung cancer so being the second leading cause of cancer deaths [13]. The majority of human malignant tumors are age-dependent cancers, with prevalence rates that exponentially increased with age. Over 75% of all invasive cancers occur in an age group of 55 years and older. Subsequently, about 80% from all breast cancer cases develop in women over the age of 50 years, producing a cumulative lifetime risk of 13.2% or 1 in 8 cases [14]. Accordantly, the current study revealed that about 87% of breast cancer patients were <40 years old. 64% of all patients have no family history of breast cancer, which probably happened due to other risk factors.

Of all breast cancer patients, 26% of them used contraceptive pills (24% were<40 and 2% were ≤ 40). Therefore, our results are in accordance with previous studies which stated that women who had ever used oral contraceptive pills had an increase in the relative risk of getting breast cancer compared to women who had never used oral contraceptive pills [15, 16].

Our results from breast cancer patients reported 38% of previous psychological problems. Therefore, these results supported some previous studies that have indicated an association between several psychological factors and a higher risk of developing cancer. In fact, over the previous 40 years, clinical and epidemiological studies have provided strong evidence for the association between depression, chronic stress and social isolation and cancer development [17].

Over the past few decades, outstanding advances have been there for breast cancer managing that lead to earlier detection for the disease, in addition to the progression of more effective treatments, which resulting in a substantial decrease in breast cancer deaths and improved results for women living with breast cancer [18]. It is no longer realized as a single disease but it is a complex disease that comprised of different biological subtypes with distinct natural history, representing a wide range of pathologic, clinical and molecular features with different prognostic and therapeutic consequences [19]. The current study revealed that the tumor incidence was higher in the left side (45%) compared to the right side (42%), then both sides (13%) with a significant difference among them (P= 0.001). These results were previously reported by Linjawi *et al.* [20].

With respect to the different clinical data, our study revealed that the positive expression was found ER, PR and HER-2 receptors with a prevalence of 90%, 73% and 28%, respectively, while the negative expression for the same receptors was found as 10%, 24% and 67% respectively. The high prevalence of ER/PR+ and low prevalence of HER2+ in our society give us hope in increasing the according to a previous study, which revealed that ER/PR+ and HER-2 had the best overall survival compared to ER/PR-, HER-2, which had the worst for both, overall survival and disease-free survival. In ER/PR+, Her2-, chemotherapy conferred better overall and disease-free survival advantages [19]. In addition, ER and HER-2, the tumor-expressed proteins play a progressively significant role in determining the appropriate breast cancer treatment. *e.g.*, ER+ breast cancer women typically receive endocrine therapy (tamoxifen or aromatase inhibitors) and Her2+ breast cancer women may receive anti-Her2 (lapatinib [Tykerb] and trastuzumab [Herceptin]) as described by Cameron *et al.* [21].

Moreover, our results revealed that invasive ductal carcinoma (IDC) is the most common type of breast cancer among all studied breast cancer patients with prevalence of 73.43% and invasive lobular carcinoma (ILC) as the second most common type (3.12%). IDC was previously reported as the most frequent type of breast cancer [22], [23].

TNM staging-based classification results revealed that the tumor stage percentages were T1 (48.40%), T2 (31.20%), T3 (14.10%) and T4 (6.20%). With significant differences among them (P= 0.001). Lymph node percentages were N0 (67.20%), N1 (28.10%) and N2 (4.70%), with significant differences among them (P= 0.001). Meanwhile, metastasis "M" percentages were M0 (95.30%) and M1 (4.70%) with a significant difference between them (P= 0.001). It was also found

that the most frequent stages of the tumor are stage I (54.68%), followed by stage III (20.31%), then stage II (18.75%) and finally stage 0 (6.25%) with significant differences (P= 0.001). These results indicated that most women were diagnosed with early stages of breast cancer, which means that they were aware of the importance of the early diagnosis for breast cancer. The disease stage is used to determine prognosis and guide management. It is also used to facilitate discussions about treatment and prognosis between collaborating providers, as well as between providers and patients [24].

The present study was carried out in order to find the association between BARD1 gene polymorphism in rs2228456, rs111367604 and rs28997576 SNPs and breast cancer through the application of tetra-primer ARMS–PCR method on 200 women (100 for each healthy woman and breast cancer patients) aged 18-82 old. This method revealed specificity and sensitivity as well as a sufficient option for the detection of SNPs [25]. A previous study has compared Tetra-primer ARMS–PCR and restriction fragment length polymorphism (RFLP) techniques. It found that both techniques yielded the same results [20]. Tetra-primer ARMS–PCR results of the current study revealed heterozygous genotype patterns for both patient and control samples using BARD1 SNP rs2228456 (A/G), while normal homozygous (non-mutant) genotypes for both patient and control samples using both BARD1 rs111367604 (C/A) and rs28997576 (C/G) SNPs. Therefore, no association was found between breast cancer and *BARD1* polymorphisms using the chosen SNPs (rs2228456, rs111367604 and rs28997576). If the sample size was larger, the study may have more chance of getting positive results and/or more SNPs should to be used to detect SNP anomalies in this gene for diagnostic purposes. A previous study found an association between *BARD1* expression and RE-negative status in breast cancer cell culture experiments. The *BARD1* levels in the breast carcinoma cells were closely linked to the progesterone receptor (PR), HER-2, histologic grade and lymph node metastases [26].

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