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TETRA-PRIMER ARMS-PCR DETECTED A MISSENSE MUTATION OF BRCA1 AND BRCA2 IN BREAST CANCER PATIENTS IN JEDDAH CONTROL STUDY

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ABSTRACT

Objective: Breast cancer is one of the major severe malignancies that affect women and occurs in either sporadic or hereditary form. The aim of this research was to study the distribution of the alleles of BRCA1 and BRCA2 genes in a sporadic breast cancer population in Jeddah in KSA. **Method:** The study included 128 participants (64 control, 64 patients). For this purpose Tetra ARMS-PCR primers technique for the detection of the missense mutation, (c.4132G>A) in exon 11 for BRCA1 and (c.1385A>G) in exon 10 for BRCA2 was employed. **Result:** The results of the study indicated that (100%) of the genotype was normal allele (GG) for BRCA1, (98.44%) of the genotype was normal allele (AA) for BRCA2 while the control had (1.56%) for hetero allele (GA). **Conclusion:** The study demonstrated that tetra ARMS-PCR method was an efficient detection for genotyping of *(BRCA1* c.4132G>A) and *(BRCA2* c.1385A>). Currently, we believe that, no sufficient proof to **conclude** that BRCA1 and BRCA2 mutation happens at a lower frequency in KSA, compared to the rest of the world. Further genetics and molecular studies are **recommended** especially those that include larger samples of breast cancer.

KEYWORDS: BRCA1, BRCA2, T-ARMS-PCR, breast cancer

INTRODUCTION

Breast cancer is one of the extremely frequent female malignancy in the world and unfortunately, its etiology remains unknown. Currently, breast cancer is believed to be a multifactorial disease that results from the interaction of environmental and genetic factors

(**Ponder, 2001**). In year 2012, there were an estimated 1.67 million new cases of breast cancer and 411,000 deaths resulting from this disease (**Ferlay *et al.*, 2015**). Breast cancer is increasing in Kingdom of Saudi Arabia (KSA) (**Hasan *et al.*, 2013**). In every year, several cases of breast cancer have been diagnosed in Saudi Arabia. It has been found that the early onset and development of breast cancer is linked to the genetic factors among the consanguineous and admixed population living in the western region of Saudi Arabia (**Merdad *et al.*, 2015**). The Saudi Cancer Registry (SCR) statistics indicate that there are approximately 5,617 females diagnosed with breast cancer with a breast cancer prevalence of about 20.6%.

BRCA1, the fists significant susceptible gene, was discovered in the past two decades in conjunction with BRCA2 they have remained the most substantial developments in breast cancer studies. Especially in the process of identification of the highly penetrant mutations in both genes have had high implications (**Zielinski, 2004**).

Both major breast cancer susceptibility genes are BRCA1 and BRCA2 located on the long arms of chromosomes 17 and 13, respectively, (**Wooster *et al.*, 1994**). Although BRCA1 and BRCA2 do not have an apparent sequence homology, they share common features. For instance, both BRCA1 and BRCA2 have an enormous exon 11 that consists of 61% and 48% of the entire coding sequence respectively. Additionally, both have a transitional start site at exon 2, and the highest levels of expressions in humans are observed in the ovaries, thymus, and testis (**Miki *et al.*, 1994; Tavtigian *et al.*, 1996**). Except for a few small domains, both genes are poorly conserved between species. When mutations occur in the BRCA1 and BRCA2 genes, the protein functions are consequently modified. As a result, this leads to a cancer predisposition phenomenon known as Hereditary Breast and Ovarian Cancer (HBOC). Moreover, both BRCA genes are autosomal dominant (**Yang and Lippman, 1999**). The mutations that occur in the BRCA1 gene cause a substantial effect on protein truncations. This may involve either small deletions, or insertions, or nonsense mutations, which leads to the formation of stop codons and at least 100 different BRCA1 missense variants. According to the documented BRCT domain, these mutations in the BRCA1 genes are characterized with structural and functional effects. However, a number of studies have shown that families with well-defined family histories of breast cancer have similar BRCA1 gene mutations in the chromosome 17 (**Chakraborty *et al.*, 2013**). In addition, the research found that the role

of BRCA1 in the development of sporadic breast cancer supported by the low expression of BRCA1 in a large rate or when loss the alleles of the gene (**Seery *et al,* 1999**).

With respect to the BRCA2 gene, the germ line inactivating mutations trigger a major proportion of genetically inherited breast cancers in both females and males. Other types of cancers include prostatic, ovarian, pancreatic, gall bladder, liver, bile duct melanoma, and stomach cancers (**Wooster *et al.*, 1994**).

El-Harith *et al* in 2002 reported 2 cases BRCA1 and BRCA2 genes mutation among 29 Arab women with breast cancer, the mutation carrier rate for both BRCA1 and BRCA2 genes was 2/29 or 7%, and that these mutations may not involve deletion. However, it is difficult to account for the unclassified variants including the polymorphism for both BRCA1 and BRCA2 genes. According to the research, the mean age of Arab with breast cancer was 41; thus, increasing the likelihood of detecting mutations of both BRCA1 and BRCA2 genes.

The aim of this study was to study the distribution of the alleles of BRCA1 and BRCA2 genes in a sporadic breast cancer population in Jeddah in KSA. The study used tetra ARMS-PCR primers technique to identify the missense mutation in the genes. In BRCA1, a single nucleotide alters the amino acid to Isoleucine (ATA) from Valine (GTC) while in BRCA2, Glutamic (GAA) is changed to Glycine (GGA).

MATERIALS AND METHODS

Human subjects

The research included 128 women from Jeddah city, with 64 of them being breast cancer patients in different stage and the rest being the control individuals. Each of them issued a written informed consent of their participation in the study about Age, Positive family history of cancer, contraceptive pills consumption, significant psychological distress and early detection of tumors by screening mammography.

Sample collection

Blood samples of participants were taken from Medical Reference Clinics and King Abdulaziz University Hospital in Jeddah. The ethical Committee (unit of biomedical ethics) did the approval of this study at King Abdulaziz University. Clinical data Classifications were based on the site, stage and type of the tumor of all patients. Genomic DNA was taken

from whole blood and stored in EDTA coated tubes (Lavender top tube, Franklin Lakes, NJ, USA).

Extraction of DNA

Genomic DNA was extracted using ReliaPrepTM Blood gDNAMiniprep System kits (PROMIGA, USA); it was taken from whole blood and stored in EDTA coated tubes (Lavender top tube, Franklin Lakes, NJ, USA). Amplification for *BRCA1* and *BRCA2* genes by Polymerase Chain Reaction (PCR) used GoTaq® Green Master Mix. First step modeling the PCR system by determination the Tetra ARMS PCR primers Tm, in this research was set to 63°C and 60°C for BRCA1, 60°C and 56°C for BRCA2. The four steps of the program were as follows:

BRCA1

Step 1 - Denaturation at 95°C for 2 min (1 cycle). Step 2 - Denaturation at 95°C for 45s, annealing at 63°C for 45s and extension at 72°C for 1 min (15 cycles). Step 3 - Denaturation at 95°C for 45s, extension at 72°C for 1 minute and annealing at 60°C for 45s (20 cycles)
Step 4 - Extension at 72°C for 5 minutes (1 cycle).

BRCA2

Step 1 - Denaturation at 95°C for 2 min (1 cycle). Step 2 - Denaturation at 95°C for 45s, annealing at 60°C for 45s and extension at 72°C for 1 min (15 cycles). Step 3 - Denaturation at 95°C for 45s, extension at72°C for 1 minute and annealing at 56 °C for 45s (20 cycles). Step 4 - Extension at 72°C for 5 minutes (1 cycle). To confirm the PCR results, 3µl of PCR product was introduced into the contents of 2% (w/v) agarose gel, the rest of PCR product stored at -20°C. The four Primer for each gene used in the study was designing by the primer Blast software (Table 1).

Statistical analysis

The statistical Package for Social Sciences (SPSS version 20) (SPSS Inc., Chicago, IL, U.S.A). Has used. Data were presented as mean +/- standard deviation or number (percentage) as appropriate. The continuous variables between two groups was made using unpaired sample "t" test and between more than two groups using OneWay ANOVA (LSD) test and between categorized data using Chi-Square test. A probability (P) <0.05 was considered significant.

RESULTS

Patients age in the study were (28 to 80 years old) comparatively older than controls (18 to 61 years old). The percentage of positive family history and early detection of tumors was higher in patients (32.80% and 12.59%) compared to controls (23.40% and 10.90%). Percentage of Contraceptive pill administration was also higher in patients (20.30%) compared to controls (1.60%). Up to 40.60% of the patients showed psychological affection. The classification of age bellow and above of 40 years old showed that (17.18%) of participants was aging ≤ 40 years old with a positive family history of cancer (27.3%), Contraceptive pills used (18.2%) and a psychological effect (27.3%). On the other hand, (82.8%) of participants was aging > 40 years old showed a positive family history of cancer (34.0%), Contraceptive pills used (20.8%) and a psychological effect (43.4%) (Table 2).

Genotyping results of the BRCA1

Figure 1, line 1-2 showed the genotyping results of BRCA1 gene in normal group. Line 3-4 showed the genotyping results of BRCA1 gene in patients group. A double bands of 321 and 121bp shows the presence of homozygous allele G (the normal gene type) in both groups.

Genotyping results of the BRCA 2

Figure 2, line 1 showed the genotyping results of BRCA2 gene in normal samples. Three bands of 628, 348 and 309bp shows the presence of heterozygous allele G and A. Line 2 showed the homozygous allele A (the normal gene type) produce a double bands of 628 and 348bp respectively in most normal group. Line 3-4 showed the genotyping results of BRCA2 gene in patients group, which was also homozygous of allele A.

Clinical and Pathological data

The tumor site, stage, and, pathological type of the tumor in patients were recorded. The tumor stages consisted of mainly stage I (54.70%), stage III (20.30%), stage II (18.80%), stage 0 (6.20%) and stage IV (4.70%) with the difference in significance (P= 0.0001). The tumor was dominant on the right side compared to the left side (39.10%) and both sides (12.50%) with a considerable difference between them (P= 0.001). In addition, the main tumor was realized to be Invasive ductal carcinoma (73.40%) than others (12.50%), Ductal carcinoma in situ (10.90%), and Invasive lobular carcinoma (3.10%) which also demonstrated a significant difference between them (P= 0.0001). The positive expression of

ER, PR, and HER2 receptors, which were (84.40%, 73.40% and 31.20%) respectively. While the negative expression were (15.60%, 26.60%, and 68.80%) respectively (Table 3).

Relationship between the hormonal receptors in different types of cancer

The relationship between the ER receptors, PR receptors and HER2 receptors, and the different types of the breast cancer demonstrated as following; For Ductal carcinoma in situ, invasive ductal carcinoma, invasive lobular carcinoma and others ductal carcinoma in situ, the ER receptors were positive (85.70%, 85.10%, 50.00% and 87.50%) respectively. In addition, the PR receptors were positive (71.40%, 74.50%, 50.00% and 75.00%) respectively; while HER2 receptors were similarly positive (28.60%, 29.80%, 50.00%, and 37.50%) respectively (Table 4).

Table 1 showed the study primer designing by primer blast software.

Gene	System	Primer sequence (5-3)	Allele	Tm©	Length	Amplicon	CG%
BRCA1	Forward outer primer F1	5' GAC ACA GCA AGT TGC AGC G 3'	-	59.5	19	321	57.89
	Reverse inner primer R2	5' CCC TGA GCA GTC TTC AGA GAC 3'	G	63.3	21	121	57.14
	Forward inner Primer F3	5' GGG TGT GAG AGT GAA ACA AGC A	A	62.1	22	242	50
	Reverse outer Primer R4	5' GTT CCA TCA AGG TGC TTA CAG TCT 3'	-	63.5	24	-	45.83
BRCA2	Forward outer primer F1	5' AAT GTA GCA AAT CAG AAG CCC3'	-	63.5	21	628	42.86
	Reverse inner primer R2	5' CTTC ATC TCTCTT ATT TAC CAC TGT T 3'	A	63.7	27	309	37.4
	Forward inner Primer F3	5' TCAGAG AAG CCATT AAA TGA GG3'	G	1.3	23	348	39.13
	Reverse outer Primer R4	5' CTG CAT TCT TCA AAG CTA CAG AA3'	-	59.3	23	-	39.13

Table 2: Demographic characteristics of participants

Data	Control (n= 64)	Patients (n= 64)	Aging ≤ 40 years old	Aging > 40 years old
Age	28.22±9.59 (18.00-61.00)	50.61±11.75 (28.00-80.00)	(n= 11)17.18%	(n= 53)82.8%
Family history of cancer	15 (23.40%)	21 (32.80%)	3 (27.3%)	18 (34.0%)
Contraceptive pills	1 (1.60%)	13 (20.30%)	2 (18.2%)	11 (20.8%)
Significant Psychological distress	-	26 (40.60%)	3 (27.3%)	23 (43.4%)
Screening	7 (10.90%)	8 (12.50%)	–	–

Table 3 Description of the stage, site and type of the tumor of all patients

Data	Patients (n= 64)	*P*- value
Stage		
0	4 (6.20%)	
I	35 (54.70%)	
II	12 (18.80%)	***P*= 0.0001**
III	13 (20.30%)	
IV	3 (4.70%)	
Site		
Right	31 (48.40%)	
Left	25 (39.10%)	***P*= 0.001**
Both sides	8 (12.50%)	
Types		
Invasive ductal carcinoma	47 (73.40%)	
Ductal carcinoma in situ	7 (10.90%)	
Invasive lobular carcinoma	2 (3.10%)	***P*= 0.0001**
Others	8 (12.50%)	
ER Positive Negative	54 (84.40%) 10 (15.60%)	***P*= 0.0001**
PR Positive Negative	47 (73.40%) 17 (26.60%)	***P*= 0.0001**
HER2 Positive Negative	20 (31.20%) 44 (68.80%)	***P*= 0.003**

Data were expressed as number (%). Significance is made using Chi-Square

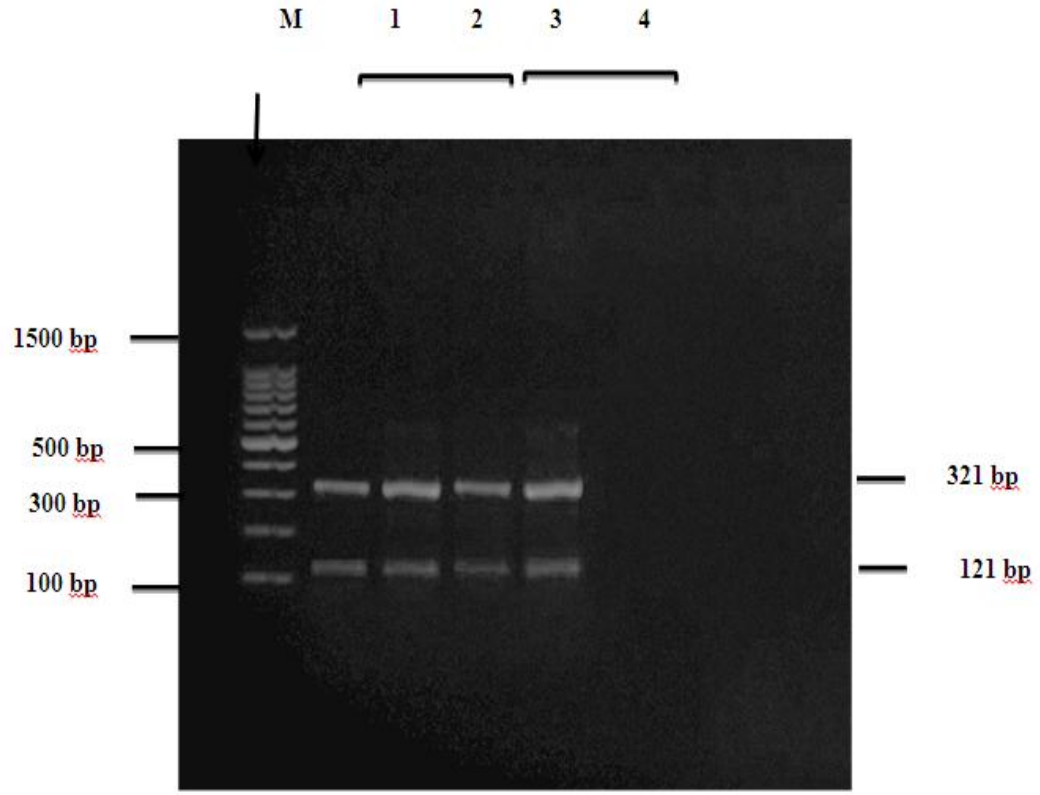

Figure 1: Photograph of a 2% (w/v) agarose gel showing the result of amplification of human exon 11 in BRCA1 gene by PCR.

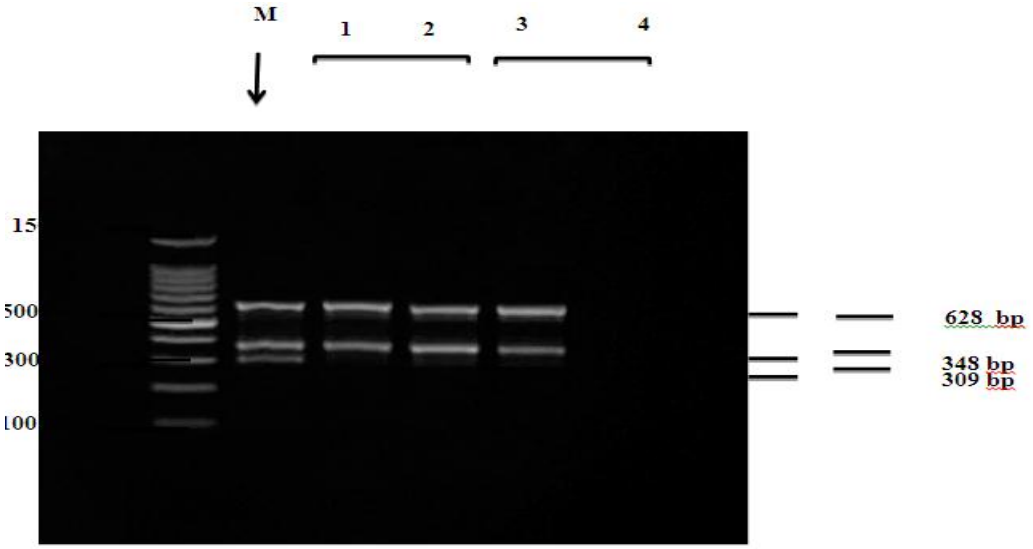

Figure 2: Photograph of a 2% (w/v) agarose gel showing the result of amplification of human exon 10 in BRCA2 gene by PCR.

Table 4: Cross tabulation of the receptors in different types of cancer.

Data	Invasive ductal carcinoma	Ductal carcinoma in situ	Invasive lobular carcinoma	Others ductal carcinoma in situ
ER				
Positive	40 (85.10%)	6 (85.70%)	1 (50.00%)	7 (87.50%)
Negative	7 (14.90%)	1 (14.30%)	1 (50.00%)	1 (12.50%)
PR				
Positive	35 (74.50%)	5 (71.40%)	1 (50.00%)	6 (75.00%)
Negative	12 (25.50%)	2 (28.60%)	1 (50.00%)	2 (25.00%)
HER2				
Positive	14 (29.80%)	2 (28.60%)	1 (50.00%)	3 (37.50%)
Negative	33 (70.20%)	5 (71.40%)	1 (50.00%)	5 (62.50%)

Data were expressed as number (%)

DISCUSSION

Breast cancer is one of the dominant diseases in developing and developed nations. In Arab nations, it is the most common diagnosed female malignant disease (**SCR, 2013**). Breast cancer incidents are lower in Arab nations compared to the USA, but the numbers are rising fast (**Chouchane *et al.*, 2013**). Both BRCA1 and BRCA2 genes have a role in the cellular processes, DNA repair and mutations in BRCA1 and BRCA2 genes resulting in breast and ovarian cancer (**Welcsh and King, 2001**). However, it is still undetermined whether BRCA1 and BRCA2 play similar important roles in sporadic breast cancer **(Yang and Lippman 1999**),

Previous studies in Saudi Arabia indicate that the genes of BRCA1 and BRCA2 mutations have minimal impact on the genetic susceptibility of breast cancer among the Arab population (**Denic and Al-Gazali, 2003**). However, King Saud University study, did not find any connection between the two genes and their potential to increase risk in Saudi population (**Hasan et al., 2013**). **Merdad *et al.*, (2015)** report was the first report published on the predisposing factors in breast cancer in Saudi Arabia population. The report demonstrated that lifestyle shifts coupled with consanguinity are the potential risk factors in early onset of breast cancer.

The result of current study showed that the average age of onset for the patients was 51 years of age and this is similar to earlier reports on age of onset in Saudi Arabia (**Ibrahim *et al.*, 2008**).

This study focused on investigating the distribution of BRCA1 alleles in exon 11 missense mutation (c.4132G>A) and BRCA2 alleles in exon 10 missense mutations (c.1385A>G)

among the women with breast cancer and compared to controls. For the purpose of the study, the Tetra ARMS-PCR technique has used to detect the emulation in the genes where a single nucleotide has changed to Isoleucine (ATC) from Valine (GTC) in BRCA1, while in BRCA2 Glutamic (GAA) has changed to Glycine (GGA). Tetra amplification refractory mutation system–polymerase chain reaction (T-ARMS-PCR) is one of the many methods developed to detect the genotype. The method utilizes primers in a single PCR followed by gel electrophoresis (**Medrano and Oliveira, 2014**).

In this study the genotype results for *BRCA1* gene in exon 11, demonstrated that 100% of genotype was the normal allele (GG) in both patients group and control group in a sporadic population of breast cancer in Jeddah, which concurred with a study on Portuguese breast cancer (**Santos and *et al,* 2014**). Both studies did not detect any association between BRCA1 (c.4132G>A) and breast cancer risk.

Exon 11 is the largest exon of the BRCA1 corresponds to 61 % of the total coding sequence (**Miki *et al.,* 1994**). Consequently, it has expected to have most of the mutations and that what expected to find in this research. Nevertheless, the result did not detect any association between BRCA1 (c.4132G>A) mutation in this exon and breast cancer risk and this may be due to the small size number of samples.[64]

On the other hand, the most common mutations were found in BRCA2 in exon 10 although exon 11is the largest exon on BRCA2 like BRCA1. The current study showed that, the genotyping result of BRCA2 in exon 10, were (100%) normal allele (AA) in patients group compared to controls (98.44%) and (1.56%) hetero allele (GA), which agreed with two studies on missense substitutions of BRCA2 (**Easton *et al.,* 2007; Tavtigian *et al.,* 2008**). Both of studies did not detect any associations between the substitutions mutation (c.1385A>G) and the risk of breast cancer, they did not find any clinical significance in relation to risk of breast cancer. In addition, **Gómez *et al.,* (2009)** had reported that the variant in BRCA2 was neutral, which agree with this study and previous studies. Current study found that (1.56%) hetero allele (GA) in control; this participant did not have a family history and suggested that the noted acquired mutation has because of exposed to the particular environment. **Wu *et al.,* (2005**) published a study demonstrated how functional assays can distinguish between neutral unknown and disease-causing variants in *BRCA2* gene. The study focuses on showing how data from genetics and functional studies could be

integrated to foretell the disease causality of BRCA2. The study discovered that the (missense mutation: c.1385A>G p. E462G) in exon 10 is a low-risk variant.

According to the literature, the relationship of BRCA1, BRCA2, and breast cancer incidents, it is highly controversial among the various ethnic populations. **Santos and Catarina, 2011** classified this variant in BRCA1 as benign. The variant of segregation in two relatives with breast cancer at early ages has considered as neutral. On the contrary, a Lebanese study concluded that breast cancer was associated with this mutation in *BRCA1* (**El Saghir *et al.*, 2015**).). The result in pervious study, leads us to suggest further investigation of the effects of these sequence variants on BRCA1 (c.4132G>A) to understand whether these variations have any pathological role.

Generally, according to clinVar whose reports of the relationships among human variations and phenotypes in BRCA1 (c.4132G>A) and BRCA2 (c.1385A>G), the Variant frequency is 0.03% (1 of 2952), 0.04% (4 of 8011) respectively (**ClinVar, 2016**).

Currently, we believe that, no sufficient proof to **conclude** that BRCA1 and BRCA2 mutation happens at a lower frequency in KSA, compared to the rest of the world. BRCA1 and BRCA2 mutations constitute only a small part of the sum cancer mutation load in the general population, and other predisposing alleles may not prove to be homozygous lethal. However, the method was used in this research tetra-primer amplification refractory mutation system polymerase chain reaction (T-ARMS-PCR) allows rapid, unexpansive, reproducible and accurate detection of mutations BRCA1 (c.4132G>A) and BRCA2 (c.1385A>G) without need of any special equipment.

Further genetics and molecular studies are **recommended** especially those that include larger samples of breast cancer predisposition. It would be enlightening to explore weather exposure to a specific environmental carcinogen, reproductive patterns; dietary and cultural practices and environmental carcinogens assimilated by women in Saudi Arabia could result in variation in mutation pattern observed in this study. Additionally, all women from age 30 should do monthly self- breast examination, regular mammograms and genetic testing.

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