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UTILITY OF AMPLIFICATION-REFRACTORY MUTATION SYSTEM FOR THE DETECTION CLINICALLY SIGNIFICANT ESTROGEN AND PROGESTERONE RECEPTORS GENE VARIATIONS IN BREAST CANCER PATIENTS IN SAUDI ARABIA

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Abstract:

Purpose:

The ER, PR genetic variations has been reported to be functional and may contribute to genetic susceptibility to Breast cancer however, their detection methods vary in sensitivity and specificity therefore molecular based assay can be potentially useful for detection of clinically important ER and PR gene variations when targeted therapies against ER,PR become available. Therefore the aim of the study was to establish economical molecular based assay for the detection of clinically important ER,PR gene variation in Breast cancer patients.

Methodology:

This study was conducted on 85 histologically confirmed Breast cancer patients. DNA was extracted by using DNA extraction kit from Breast cancer patient and ARMS-PCR was established for the detection estrogen and progesterone receptor gene variations in Breast cancer patients. The results were confirmed by Sanger sequencing.

Results:

Of 85 consecutive breast cancer patients, (27 % were below or equal to 40 years age and 73% were above 40 years of age. Of breast cancer cases 38 (44.70%) were in early (I &II) stage and 47 (55.29%) cases were in advanced stages (III & IV). Out of 100 cases, 69 (81%) patients had distant metastasis and 16 (19%) does not show distant metastasis and out of 85 cases, 9 (11 %) patients were treated with herceptin and 76 (89 %) were not treated with herceptin. Tamoxifen has been the basis of endocrine therapy for patients with ER (+) breast cancer for more than three decades. The treatment reduces the annual mortality rate of breast cancer by 31%, and remains the most effective targeted cancer therapy. However, approximately one-third of patients treated with adjuvant tamoxifen suffer from aggressive recurrent disease. Resistance to tamoxifen, thus, remains a major challenge in providing effective treatments for these patients. There are many conflicting results in the literature comparing quality of life following breast-conserving therapy (BCT) and mastectomy. In our study, out of 85 cases, 29 (34 %) patients received mastectomy and 56 (66%) did not received mastectomy. Lumpectomy (also known as breast conserving surgery, partial mastectomy or wide excision) is a surgery to remove cancer from the breast. Unlike a mastectomy, a lumpectomy removes only the tumor and a small rim of normal tissue around it. It leaves most of the breast skin and tissue in place. In our study, out of 85 cases, 17 (20%) patients received mastectomy and 68 (80%) did not received mastectomy. Amplification-refractory mutation system PCR for estrogen receptor 1 (ESR1 PvuII-rs2234693 T>C) and progesterone receptor (PR) rs590688 G/C, rs10895068C/T gene variations was successfully was established in Breast cancer.

Conclusion:

It was concluded that the ARMS-PCR assay proved to an appropriate methodology considered for using ER and PR gene variations and allows discrimination between homozygous and heterozygous individuals. The test is therefore a simple, fast, and inexpensive procedure that does not entail any special equipment other than a thermocycler.

Keywords:

Breast cancer, Estrogen and progesterone receptors, ESR1 PvuII gene variation -rs2234693 T>C , progesterone receptor, +331 C/T, rs10895068, Amplification Refractory Mutation System (ARMS).

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INTRODUCTION:

Breast cancer is the most common cancer among women globally and leading cause of cancer deaths among women worldwide and the second most common malignancy among females in Middle east countries and the Kingdom of Saudi Arabia (KSA) is no exception(1) .Breast cancer carries tremendous socio-economic, emotional, and public health implications. More than one million new cases of Breast cancer are diagnosed annually. Breast cancer incidence rates in Arab women have increased during the last 25 years. Globally, over the last few decades there have been outstanding advances in breast cancer management leading to earlier detection of disease and the development of more effective treatments resulting in significant declines in breast cancer deaths and improved outcomes for women living with the disease, especially in developed countries (2) but in Saudi Arabia women are still being diagnosed with Breast cancer at more advanced stages of the disease. It has been reported that the morbidity and mortality have been shown to be effectively reduced by early detection of BC through screening activities (3). Despite this finding, low participation rates in BC screening activities have been reported among Saudi Arabian women (4). Breast cancer is reported from developed nations and Western countries, while data on relevant reports from KSA appears either scattered, or not brought to the limelight (6).Breast cancer histogenesis is related to cells of either epithelial origin in tubules/ducts or mesenchymal cells. Extensive research suggests that genetic, hormonal and environmental factors may possibly be related to its aetiology (7).The predictive factors predict response to treatment whereas prognostic factors are those which determine the outcome of disease in the absence of treatment (8). Thus, among other prognostic indicators for breast cancer, studies have shown that the presence of estrogen and progesterone receptors and HER-2 proteins to have influence in the prognosis of patients with breast cancer (9).The presence of estrogen (ER) in particular as well as progesterone (PgR) receptors is important clinically, as a predictor of response to adjuvant hormonal therapy rather than prognostic factors (10).

Role of estrogen and progesterone receptors:

Estrogen receptors (ER) and progesterone receptors (PR; also called PgR) may be found in breast cancer cells. Cancer cells with these receptors depend on estrogen and related hormones, such as progesterone, to grow. Estrogen and progesterone influence many hormonal functions in women, such as breast development. If breast cancer cells have estrogen receptors, the cancer is called ER-positive breast cancer. If breast cancer cells have progesterone receptors, the cancer is called PR-positive breast cancer (11). If the cells do not have either of these two receptors, the cancer is called ER/PR-negative. About two-thirds of breast cancers are ER and/or PR positive. Testing the tumor for both estrogen and progesterone receptors is a standard part of a breast cancer diagnosis. Because the results are used to guide treatment, it's important that the results be accurate (12). The most common method currently used to test a tumor for estrogen and progesterone receptors is called immunohistochemistry or IHC. IHC testing can detect estrogen and progesterone receptors in cancer cells from a sample of tissue. This tissue may come from a biopsy, or from the surgery to remove all of the tumor and some or all of the breast.ER and PR are intracellular steroid hormone receptors which have received substantial attention since 1986. Measurable amounts of ER and PR are found in about 50–86% of patients with breast cancer (13).The majority of breast cancers show overexpression of estrogen receptors (ERs) and progesterone receptors (PRs). The development of drugs to target these hormone receptors, such as tamoxifen, has brought about significant improvement in survival for women with hormone receptor-positive breast cancers (14). Studies of primary chemoprevention with tamoxifen have generated high expectations and considerable success rates. The efficacy of lower doses of tamoxifen is similar to that seen with a standard dose of the drug, and there has been a reduction in healthcare costs and side effects (15). Since information about ER and PR is vital for patient management, quality assurance is

important to ensure accurate testing. In recent guidelines, the recommended definition of ER and PR positivity is 1% or more of cells that stain positive. Semiquantitative assessment of ER and PR is important for prognosis and, hence, management. Even with the development of genomic tests, hormone receptor status remains the most significant predictive and prognostic biomarker (16). *ESR1* mutations are significantly enriched in endocrine therapy-resistant, metastatic breast cancer and are rare or nonexistent in treatment-naïve, primary tumors. Based on published reports, the overall frequency of ER mutations in metastatic, ER-positive breast cancers ranged from 11 to 54.5%, depending on the clinical characteristics of the cohort and the method of identification (17-20). The progesterone receptor (PgR), which is located on chromosome 11q22 and is a member of a large ligand activated nuclear receptor family characterized by a central DNA binding domain, a carboxyl-terminal ligand binding domain and multiple activation (AF) and inhibitory (IF) functional elements (21-22). Progesterone, a steroid hormone, binds to the PgR, causing dimerization and thereby activating its target genes to transcribe through its response elements (PRE). The binding and activation are responsible for the mammary epithelial proliferation (23). The PgR gene is transcribed by two alternative promoters that translate into two isoforms, progesterone receptor A (PgRA) and progesterone receptor B (PgRB) through which the PgR mediates its physiological functions. PROGINS, a PR polymorphic variant, affects progesterone receptor A & B (PRA and PRB) and acts as a risk-modulating factor in several gynaecological disorders. In PgR gene six variable sites have been detected, four polymorphism and five common haplotypes (24). The most widely studied polymorphism in the promoter region of the PgR gene is the C to T substitution at position +331 (progesterone receptor, +331 C/T, rs10895068, +331 from ATG start codon). This SNP in the promoter of *PR* (+331 C/T; rs10895068) creates a unique start site which increases transcription of the PR-B isoform and has been associated with increased risk for developing endometrial cancer (25).The Amplification Refractory Mutation System (ARMS) is an application of PCR in which DNA is amplified by allele specific primers. In PCR mismatch at the 3' end of the primer can dramatically reduce the annealing and hence the amplification. This is due to the absence of 3' to 5' exonuclease proofreading activity of Taq polymerase. High fidelity DNA polymerases, that have this activity, cannot be used in ARMS. It is an extremely useful method for identification of point mutations or polymorphisms. Since the ARMS-PCR is mostly done to identify a mutation or a polymorphism it is also important that it should be able to identify whether the change in DNA is heterozygous or homozygous. Molecular based assay to be established can be potentially useful for detection clinically important ER and ER gene variations when targeted therapies against ER,PR become available. Therefore the aim of the study was to establish rapid, potentially useful and economical molecular based assay for the detection of clinically important gene variation in Breast cancer patients.

MATERIAL AND METHODS:

Study Population

This study was done on 85 histologically confirmed Breast cancer patients .This research study was approved by the Research ethics committee of University of Tabuk and written informed consent was obtained from all the subjects before enrollment.

Sample collection

Blood samples were collected from Breast cancer patients as well as from sex matched health controls in EDTA tubes after obtaining a written informed consent form. Patients with any previous history of cancer were excluded from this study. After assessing the clinicopathological findings, a 3ml sample of peripheral blood was collected by venipuncture in EDTA tubes from each patient. Also 3ml sample of peripheral blood was collected from each and every sex matched healthy controls.

DNA Extraction

Genomic DNA was extracted from peripheral blood leukocytes using QIAmp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's instructions from Breast cancer patients and sex matched health controls. The extracted DNA was dissolved in nuclease-free water and stored at 4° C until use. Quality and integrity of DNA were checked by NanoDrop™ (Thermo Scientific, USA).

The amplification-refractory mutation system (ARMS)

The amplification-refractory mutation system (ARMS) is a simple method for detecting any mutation involving single base changes or small deletions. ARMS is based on the use of sequence-specific PCR primers that allow amplification of test DNA only when the target allele is contained within the sample. Following an ARMS reaction the presence or absence of a PCR product is diagnostic for the presence or absence of the target allele .It is a simple method for detecting any mutation involving single base change.

Optimization of ARMS-PCR for estrogen receptor 1(ESR1 PvuII gene variation -rs2234693 T>C)

ESR1 PvuII (rs2234693 T>C) gene polymorphism was studied by optimizing ARMS-PCR .The primers were designed by using primer3 software as depicted in Table 1.The gradient PCR was performed in a reaction volume of 25uL containing template DNA (50ng), Fo-0.25uL, Ro-0.25uL of 25pmol of each primers ,FI-0.25uL, RI-0.25uL of 25pmol of each primers and 10uL from GoTaq® Green Master Mix (cat no M7122) (Promega, USA). The final volume of 25uL was adjusted by adding nuclease free ddH_2O. Finally, the 2ul of DNA (50ng) was added from each patient.

Table No 1: ARMS primers for the genotyping of Estrogen receptor-1 (ESR1 PvuII -rs2234693 T>C)

Direction	Sequence of primer	PCR product	Annealing temperature
ESR1-$F_0$1	5'-TGATATCCAGGGTTATGTGGCAA-3'	278bp	58^0C
ESR1-$R_0$1	5'-CTGCACCAGAATATGTTACCTATAAAAA-3'		
ESR1-FI2 C	5'-TGAGTTCCAAATGTCCCAGCC-3'	193bp	
ESR1-RI2 T	5'-GGGAAACAGAGACAAAGCATAAACA-3'	131bp	
Fo-outer forward primer :Ro-Reverse outer primer :AT-annealing temperature ,FI-Inner forward primer :RI-Inner Reverse primer			

Thermocycling conditions:

The cycling conditions comprised a hot start at 95°C for 10 min, followed by 35 amplification cycles at 95°C for 35 seconds, 58°C for 40 seconds, and 72°C for 45 seconds followed by one elongation step at 72°C for 10 min.

Optimization of amplification-refractory mutation system (ARMS) for progesterone receptor (*PR*) rs590688 G/C gene polymorphism

Minor allele frequencies of rs590688 CC PGR gene polymorphism has been reported to be significantly higher in breast cancer patients compared to controls. Progesterone receptor rs590688 G/C gene polymorphism was studied by optimizing ARMS-PCR .The primers were designed by using primer3 software as depicted in Table 2.The gradient PCR was performed in a reaction volume of 25uL containing template DNA (50ng), Fo-0.25uL, Ro-0.25uL of 25pmol of each primers ,FI-0.25uL, RI-0.25uL of 25pmol of each primers and 10uL from GoTaq® Green Master Mix (cat no M7122) (Promega, USA). The final volume of 25uL was adjusted by adding nuclease free ddH_2O. Finally, the 2ul of DNA (50ng) was added from each patient.

Table No 2: ARMS primers for the genotyping of progesterone receptor (PgR) rs590688 C/G (C Minor allele)

Direction	Sequence of primer	PCR product	Annealing temperature
PGRFo1	5'-ATAGTACAATCAGAGTGCAGCAGAGTGC-3	281bp	60°C
PGRR01	5'-CTGACCACTTTGGTTTAAGCCACTCTTA-3		
PGR FI2 G	5'-GTACCACTGGAGACACTGGAGCAGAGGG-3	195bp	
PGRRI2C	5'-CTGTTAAAATATAAGTCAGATCATGTTTGG-3	144bp	

Thermocycling conditions:

The cycling conditions comprised a hot start at 95°C for 10 min, followed by 35 amplification cycles at 95°C for 35 seconds, 60°C for 40 seconds, and 72°C for 45 seconds followed by one elongation step at 72°C for 10 min. The amplification products were separated by electrophoresis through 2% agarose gel stained with 0.5µg/mL ethidium bromide and visualized on a UV transilluminator.

Optimization of MS-PCR for progesterone receptor (*PR*) rs10895068C/T gene polymorphism

PROGINS, a haplotype of progesterone receptor consisting of 306-bp insertion in intron G together with point mutations in exon 4 and 5 is associated with reduced amounts of gene transcript and lesser response to progesterone. Since these three mutations always occur together and are in complete linkage disequilibrium, detection of 485 bp insertion in intron G is used to identify progin allele. Mutation specific –PCR was establishing by designing specific primers using primer3 software as depicted in table 3.

Table 3 : Mutation specific primer sequence for progesterone receptor (*PR*) rs10895068C/T gene polymorphism				
Direction	Primer sequence	Annealing temperature	C Allele	T Allele
Forward	5-'TATGAGCTATTTGAGTAAAGCCT-3'	55°C	185 bp	485 bp
Reverse	5'-TTCTTGCTAAATGTCTGTTTTAA-3'			

The PCR was performed in a reaction volume of 25uL containing template DNA (50ng), F -0.25uL , R-0.25uL of 25pmol of each primers and 10uL from GoTaq® Green Master Mix (cat no M7122) (Promega, USA). The final volume of 25 uL was adjusted by adding nuclease free ddH_2O. Finally, the 2ul of DNA (50ng) was added from each patient. The cycling conditions comprised a hot start at 95°C for 10 min, followed by 35 amplification cycles at 95°C for 30 seconds, 55°C for 55 seconds, and 72°C for 45 seconds followed by one elongation step at 72°C for 10 min.

Confirmation of results of progesterone receptor polymorphism by Sequencing: To confirm the mutation of progesterone receptor (*PR*) gene detected by mutation specific PCR, 20 randomly selected PCR products from the PCR systems for polymorphic sites in exon 4 and 5 were sequenced using an sanger sequencing .Two primers F seq and R Seq were used as sequencing primers for the detection of the progesterone receptor gene mutation as depicted in table 4 .

Table 4 : Sequencing primers for progesterone receptor (*PR*) rs10895068 C/T gene polymorphism			
Direction	**Primer sequence**	**Product size**	**Annealing temperature**
F sequence	5-CTTCTGTCCGAGGACTGGAG-3	211bp	60
R sequence	5-CTGTACCGAGAGGTCCGACTAG-3		

The PCR was performed in a reaction volume of 25uL containing template DNA (50ng), F -0.25uL , R-0.25uL of 25pmol of each primers and 10uL from GoTaq® Green Master Mix (cat no M7122) (Promega, USA). The final volume of 25 uL was adjusted by adding nuclease free ddH_2O .Finally; 2ul of DNA (50ng) was added from each patient. The cycling conditions comprised a hot start at 95°C for 10 min, followed by 35 amplification cycles at 95°C for 30 seconds, 60°C for 35 seconds, and 72°C for 40 seconds followed by one elongation step at 72°C for 10 min. The amplification products were separated by electrophoresis through 2% agarose gel stained with 0.5µg/mL ethidium bromide and visualized on a UV transilluminator. The PCR amplification generated a PCR product of 211 bp followed by purification using QIAquick PCR Purification Kit from Qiagen (Germany). Finally the purified PCR products were sent for sequencing to Agrigenome Company (India) http://www.aggenome.com/ as depicted in the figure 2 and 3.

RESULTS:

Demographical profile of the enrolled Breast Cancer Patients

The clinicopathological characteristics of breast cancer patients are depicted in table 5.Of 85 consecutive breast cancer patients, 23 (27 %) were below or equal to 40 years age and 62 (73%) were above 40 years of age. Of breast cancer cases 38 (44.70%) were in early (I &II) stage and 47 (55.29%) cases were in advanced stages (III & IV). Histological grading of the patient tumor showed that 07 (8%), 35 (41%) and 43 (51%) were in grade I, II and III respectively. Out of 100 cases, 69 (81%) patients had distant metastasis and 16 (19%) does not show distant metastasis. Out of 85 cases, 9 (11 %) patients were treated with herceptin and 76 (89 %) were not treated with herceptin. Tamoxifen has been the basis of endocrine therapy for patients with ER (+) breast cancer for more than three decades. The treatment reduces the annual mortality rate of breast cancer by 31%, and remains the most effective targeted cancer therapy. However, approximately one-third of patients treated with adjuvant tamoxifen suffer from aggressive recurrent disease. Resistance to tamoxifen, thus, remains a major challenge in providing effective treatments for these patients.

Out of 85 cases, 35 (41%) patients were treated with Tamoxifen and 50 (59%) were not treated with Tamoxifen. There are many conflicting results in the literature comparing quality of life following breast-conserving therapy (BCT) and mastectomy. In our study, out of 85 cases, 29 (34 %) patients received mastectomy and 56 (66%) did not received mastectomy. Lumpectomy (also known as breast conserving surgery, partial mastectomy or wide excision) is a surgery to remove cancer from the breast. Unlike a mastectomy, a lumpectomy removes only the tumor and a small rim of normal tissue around it. It leaves most of the breast skin and tissue in place. In our study, out of 85 cases, 17 (20%)

patients received mastectomy and 68 (80%) did not received mastectomy.

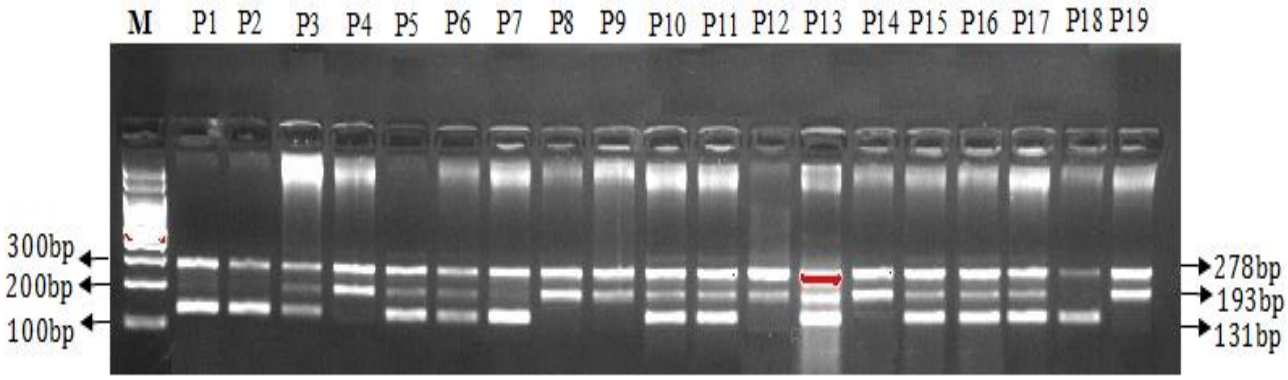
Table 5: Clinicopathological Characteristics of Breast Cancer Patients

Parameters	N=85	%
Age Group		
Age >40	62	73%
Age≤40	23	27%
Stage status		
Early (I & II)	38	44.70%
Advanced (III & IV)	47	55.29%
Grade I vs Grade II		
Grade I	7	8.23%
Grade II	35	41.17%
Grade III	43	50.58%
Estrogen receptor status		
Positive	33	39%
Negative	52	61%
Progesterone Receptor status		
Positive	59	69%
Negative	26	31%
Her2/neu status		
Positive	33	39%
Negative	52	61%
Distant Metastasis status		
Positive	69	81%
Negative	16	19%
Herceptin treatment		
Herceptin	**9**	11%
No Herceptin	**76**	89%
Tamoxifen treatment		
Tamoxifen	35	41%
No Tamoxifen	50	59%
Mastectomy treatment		
Mastectomy	29	34%
No Mastectomy	56	66%
Lumpectomy Surgery		
Lumpectomy	17	20%
No Lumpectomy	68	80%

ARMS-PCR for estrogen receptor 1(ESR1 PvuII gene variation -rs2234693 T>C) :

ESR1 PvuII (rs2234693 T>C) gene polymorphism was optimized by gradient PCR .The amplification products were separated by electrophoresis through 2% agarose gel stained with 0.5µg/mL ethidium bromide and visualized on a UV transilluminator. Primers FO and RO flank the intron of the PgR rs590688 C/G, resulting a band of 278 bp to act as a control for DNA quality and quantity. Primers Fwt and RO amplify a wild-type allele (T allele), generating a band of 131 bp, and primers FO and Rmt generate a band of 193 bp from the mutant allele (C allele) as depicted in figure 1.

Figure-1 Genotyping *of* PvuII (rs2234693 T>C) gene *polymorphism of ESR1* by amplification refractory mutation system (ARMS)-PCR in Breast cancer patients



Legends

M -100 bp DNA ladder

Homozygous TT genotype – P1,P2 and P18

Homozygous CC genotype - P8 ,P9,P19

Heterozygous T/C genotype-P3,P5,P6,P10,P11,P13,P15,P16 and P17

Control band -278bp

TT genotype-131bp

CC- genotype-278bp

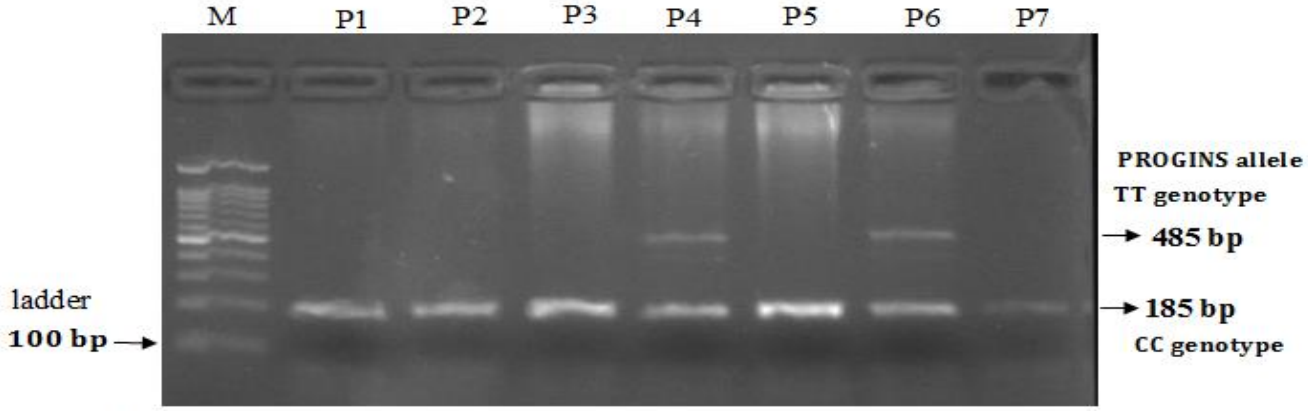
Detection of Progesterone receptor (*PR*) rs10895068C/T gene polymorphism by AS-PCR:

Progesterone receptor (*PR*) rs10895068C/T gene variability was optimized by gradient PCR .The amplification products were separated by electrophoresis through 2% agarose gel stained with 0.5µg/mL ethidium bromide and visualized on a UV transilluminator. Two DNA fragments could be detected: a 185 bp DNA fragment representing 'CC genotype or C allele (wild-type) 185 bp DNA fragment and a 485 bp DNA fragment representing 'TT genotype or T allele (mutant-type) as depicted in the figure 2.

Confirmation of Progesterone receptor (*PR*) rs10895068C/T gene mutation by sequencing:

To confirm the progesterone receptor rs10895068C/T gene mutation by sequencing, 20 randomly selected PCR products from the PCR systems for polymorphic sites in exon 4 and 5 were sequenced using an sanger sequencing as depicted in the figure 3 & 4. .Two primers F seq and R Seq were used as sequencing primers for the detection of the progesterone receptor (*PR*) gene mutation. The amplification product of 211bp was separated by electrophoresis through 2% agarose gel stained with 0.5µg/mL ethidium bromide and visualized on a UV transilluminator. The amplified product of 211bp has been purified and then sent for sequencing.

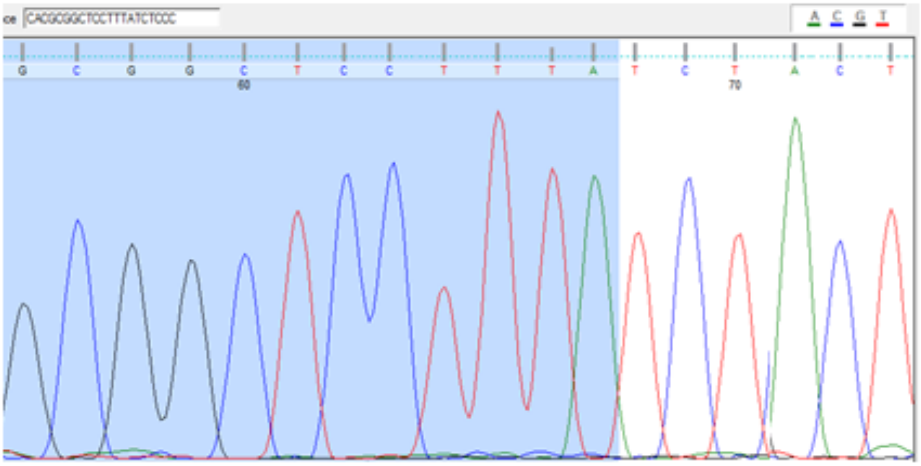
Figure 2. Optimization of mutation PCR for progesterone receptor (*PR*) rs10895068C/T gene mutation



Legend
M-100 bp ladder
Homozygous CC- P1, P2, P3, P5 &P7
Homozygous TT-
Heterozygous C/T- P4, P6

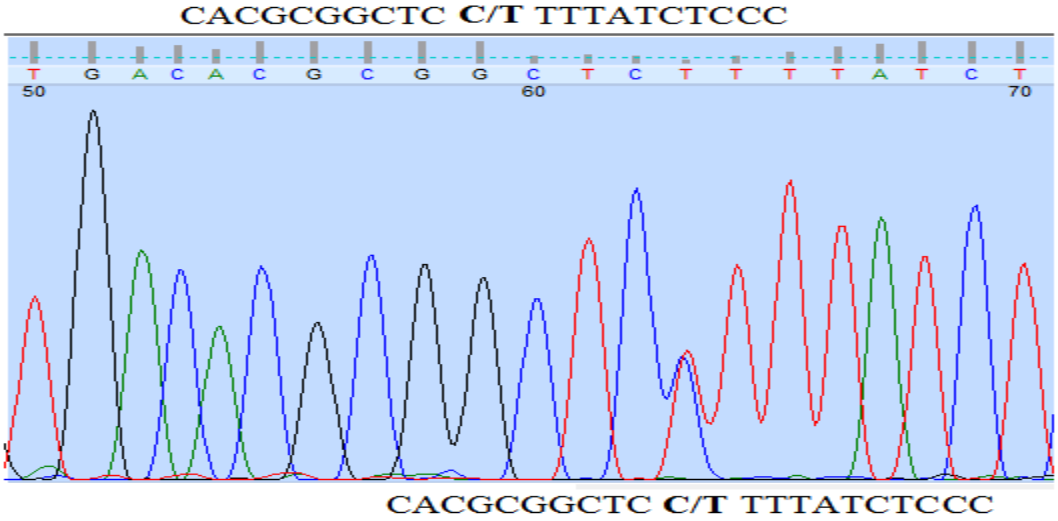
Figure 3. Sequencing results of PgR rs10895068 CC genotype (homozygous CC)

CACGCGGCTC**C**TTTATCTCCC



CACGCGGCTC**C**TTTATCTCCC

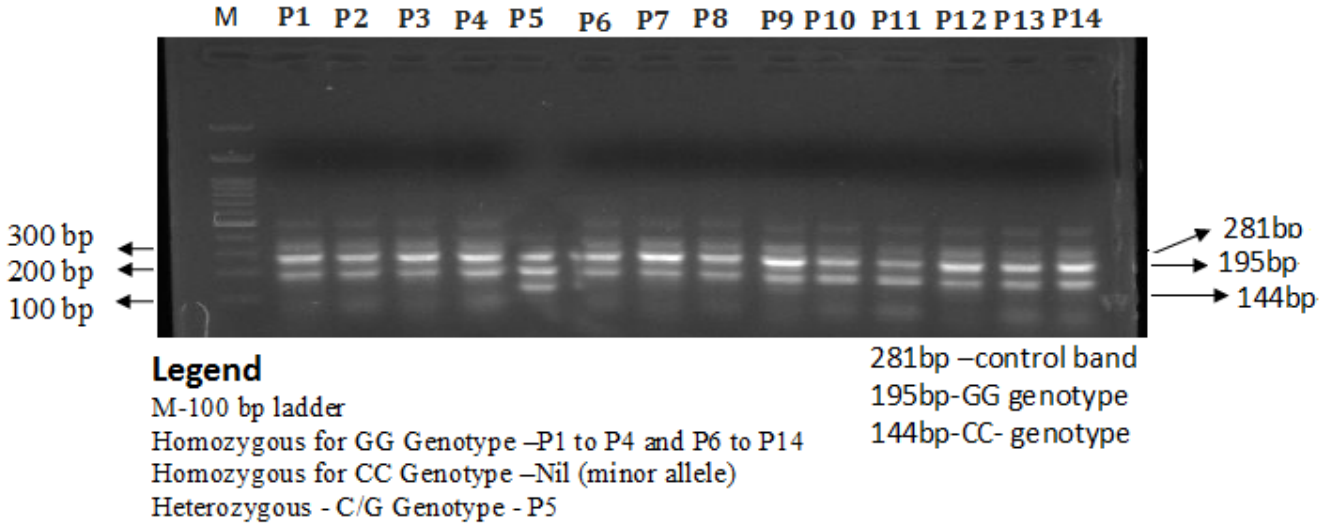
Figure 4. Sequencing results of PgR rs10895068 C/T genotype (heterozygous C/T)



Detection of progesterone receptor (*PR*) rs590688 G/C gene polymorphism by Amplification-refractory mutation system (ARMS).

ARMS-PCR for for progesterone receptor (*PR*) rs590688 G/C gene polymorphism was optimized by gradient PCR .The amplification products were separated by electrophoresis through 2% agarose gel stained with 0.5µg/mL ethidium bromide and visualized on a UV transilluminator. Primers FO and RO flank the SNP site of the PgR rs590688 C/G, resulting a band of 281 bp to act as a control for DNA quality and quantity. Primers Fwt and RO amplify a wild-type allele (C allele), generating a band of 195bp, and primers FO and Rmt generate a band of 144 bp from the mutant allele (C allele) as depicted in Figure 5.

Figure 5. Optimization of amplification-refractory mutation system (ARMS) for progesterone receptor gene variation (PgR rs590688 G/C) in Breast cancer patients



DISCUSSION:

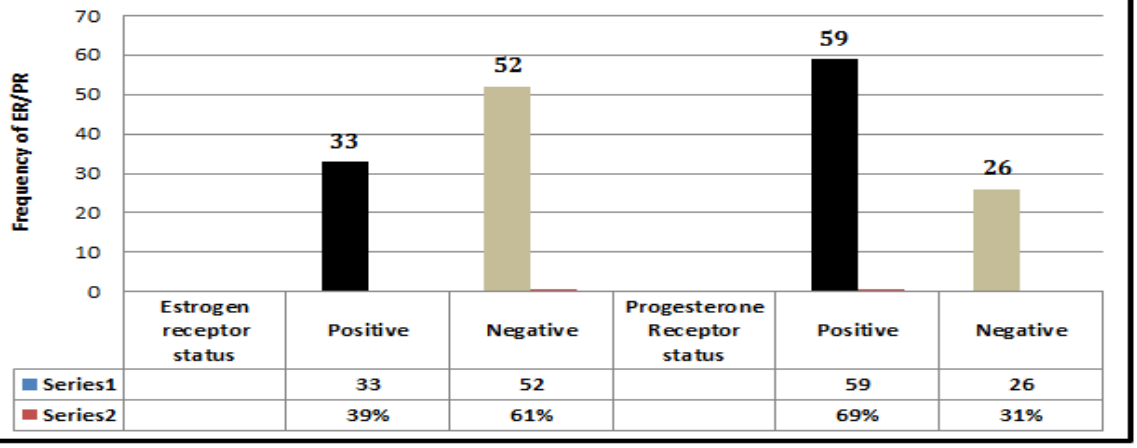
Breast cancer is a multifactorial disease and its pathogenesis is not yet fully understood. Recent studies have shown that the underlying mechanisms of breast cancer are composed of both genetic and environmental factors, and that certain SNPs within inherited susceptibility genes might exert their effects on breast cancer development. About 1.3 million American women annually are diagnosed with breast cancer and about 465 000 die from the disease

According to the American Cancer Society, (26). Similarly to other countries, breast cancer in Saudi Arabia is the most common cancer in women (27). It was reported by The Saudi National Cancer Registry a rising proportion of breast cancer among women of all ages, from 10.2% in 2000 to 24.3% in 2005 (28). In Arab countries the average age at presentation of breast cancer is 48 years, which is a decade earlier than in western countries (29). Several studies reported that the median age of onset of breast cancer among Saudi women is 46 years (30). Due to the increasing incidence, several articles have been published on screening for breast cancer and on public awareness programs initiated by the Saudi Arabian government and non-governmental sectors (31-32). We have collected 85 consecutive breast cancer patients, 23 (27 %) were below or equal to 40 years age and 62 (73%) were above 40 years of age. The incidence of breast cancer cases diagnosed in advanced stage was 55.29% that higher than the early stage cases. Tamoxifen has been the basis of endocrine therapy for patients with ER (+) breast cancer for more than three decades. The treatment reduces the annual mortality rate of breast cancer by 31%, and remains the most effective targeted cancer therapy. However, approximately one-third of patients treated with adjuvant tamoxifen suffer from aggressive recurrent disease. Resistance to tamoxifen, thus, remains a major challenge in providing effective treatments for these patients. Out of 85 cases, 35 (41%) patients were treated with Tamoxifen and 50 (59%) were not treated with Tamoxifen. There are many conflicting results in the literature comparing quality of life following breast-conserving therapy (BCT) and mastectomy. In our study, out of 85 cases, 29 (34 %) patients received mastectomy and 56 (66%) did not received mastectomy. Lumpectomy (also known as breast conserving surgery, partial mastectomy or wide excision) is a surgery to remove cancer from the breast. Unlike a mastectomy, a lumpectomy removes only the tumor and a small rim of normal tissue around it. It leaves most of the breast skin and tissue in place. In our study, out of 85 cases, 17 (20%) patients received mastectomy and 68 (80%) did not received mastectomy.

Receptor status in Breast cancer

Based on the receptor status, out of 100 Breast cancer cases 33 (39%) were positive for Her2/neu, 33 (39%) were carrying estrogen receptor and 59 (69%) were +ve for progesterone receptor.

Figure 6: Status of progesterone and estrogen receptor in breast cancer patients



Breast cancer is a partially heritable trait and genome-wide association studies (GWAS) have identified over 180 common genetic variants associated with breast cancer. Genome-wide association studies (GWAS) have identified over 180 common single nucleotide polymorphisms (SNPs) associated with risk of breast cancer (33). The majority of these SNPs were identified in European ancestry and East Asian ancestry populations, although some unique SNPs have been identified in African American populations (34) and in Latina populations (35).Several GWAS studies have identified SNPs at 6q25 that are associated with breast cancer risk (36) and mammographic density (37). The initial report identified a SNP in the intergenic region between *ESR1* and *CDCC170* in an East Asian population (38). Our lab reported the importance of ARMS-PCR system for the detection of different gene polymorphisms in Breast Cancer patients. The technique proved to be economical

rapid and simple (39) and reported a significant relationship between the different gene polymorphisms BRCA1-3'UTR germline variant rs8176318G>T and susceptibility to Breast cancer in an ethnic population of Saudi Arabia. (40)The locus was then confirmed in other populations and several additional variants were identified More recently, a fine-mapping and functional approach at this locus identified five distinct common variants associated with risk of different subtypes of breast cancer (41).We have improved the "polymerase chain reaction" (PCR) to permit rapid analysis of any known mutation in genomic DNA. We demonstrate a system, ARMS (Amplification Refractory Mutation System), for studying the pprogesterone and estrogen receptor gene variations like rs10895068C/T , ESR1 PvuII -rs2234693 T>C and progesterone receptor (*PR*) rs590688 G/C gene that allows genotyping solely by inspection of reaction mixtures after agarose gel electrophoresis. We find the ARMS-PCR system is simple, reliable and non-isotopic. It clearly distinguishes heterozygotes at a locus from homozygotes for either allele. ARMS system neither requires restriction digestion enzyme. Also ARMS system was much better than allele specific PCR in many ways. ARMS-PCR system is based on the invention that unexpectedly, oligonucleotides with a mismatched 3′-residue will not function as primers in the PCR under appropriate conditions. We have analyzed ER and PR gene variations from Breast cancer patients and sex matched from normal individuals. We confirmed our results with Sanger sequencing and our findings are in complete agreement with allele assignments derived by direct sequencing of PCR products. Following an ARMS reaction the presence or absence of a PCR product is diagnostic for the presence or absence of the target allele. The protocols detailed here outline methods that can be used to analyze human genomic DNA for one or more mutations. Since the ARMS PCR is mostly done to identify a mutation or a polymorphism it is also important that it should be able to identify whether the change in DNA is heterozygous or homozygous. A heterozygote or homozygote is differentiated by using ARMS primers for the mutant/polymorphic and the normal (wild type) alleles. The reactions for the mutant and the normal alleles are usually carried out in separate tubes. But these may be done in the same tube after labeling the two primers with different fluorescent dyes. The sensitivity and specificity of an ARMS reaction can be controlled by stringent reaction conditions. Good primer design, higher annealing temperature and limited number of cycles are important in avoiding false results (41).The number of cycles should be just enough to give a clear positive result. Increasing the number of cycles un-necessarily can cause false positives. The usual length of ARMS primer is 30 bases. Primers of this length have a high Tm and annealing temperature and are therefore more specific. The amplification refractory mutation system (ARMS) proved to be simple method for detecting any polymorphism or mutation involving single base changes or small deletions and is based on the use of sequence specific PCR primers that allow amplification of test DNA only when the target allele is contained within the sample.

Moreover, the data show that direct sequencing is not an adequate technique for polymorphism identification; therefore, appropriate methodology should be considered for using this molecular marker in the process of diagnosis. It has been have demonstrated that this high sensitivity is not accompanied by any significant compromise in the diagnostic specificity of the assay. In addition, the ARMS-PCR assay shows different patterns for normal, mutant, or mixed genotypes, minimizing the chance of misinterpretation (42). With simply one tube of PCR reactions and obviation for special equipment and reagents, ARMS-PCR represents a time- and labor-saving as well as cost-efficient method that is readily and broadly applicable in clinical molecular diagnostic laboratories.

CONCLUSION:

It was concluded that the ARMS-PCR assay proved to an appropriate methodology considered for using ER and PR gene variations and allows discrimination between homozygous and heterozygous individuals. The test is therefore a simple, fast, and inexpensive procedure that does not entail any special equipment other than a thermocycler.

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DISCLOSURE:

This manuscript is not under consideration by any other publication and has not been published elsewhere. Authors have declared that no competing interests exist.

CONSENT:

All authors hereby declare that all experiments have been examined and approved by the Research ethics committee, University of Tabuk, and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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