



**Faculty of Pharmacy, Nursing and Health Professions  
Master Program in Clinical Laboratory Sciences**

**Hereditary Breast Cancer caused by germline mutations in BRCA1  
and BRCA2 genes in Palestinian women**

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**2019**



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سرطان الثدي الوراثي و التوصيف الجزيئي للجينات المرتبطة بها (BRCA1 ,BRCA2)  
وانتشارها بين النساء الفلسطينيات

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the Masters Degree in Clinical Laboratory Science from the Faculty of  
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*I dedicate this dissertation to my mother's soul who lost her struggle with breast cancer, my sincere and loving husband Saba, my daughters Joyce, Levana and Crystal and my son David.*

*Without your support I would not be where I am today*

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Thank you very much everyone

## **Declaration**

I certify that the work provided in this thesis, unless otherwise referenced, is my own work, and to the best of my knowledge and belief , it has not been submitted elsewhere for any other degree or qualification.

I understand the nature of plagiarism, and I am aware of the University's policy on it.

**N J**

*Nancy jilleh*

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## List of Abbreviations

BC	Breast cancer
DCIS	Ductal Carcinoma in Situ
LCIS	Lobular Carcinoma In Situ
HR	Homologous Recombination
BRCA1	Breast Cancer Susceptibility Gene 1
BRCA2	Breast Cancer Susceptibility Gene 2
ER	Estrogen receptor
PR	Progesterone receptor
HBOC	Hereditary Breast and Ovarian Cancer
DsDNA	Double strand DNA
BRCT	C- terminus domain of BRCA1 gene
PAR	Population attributable risk
OCCR	Ovarian cancer cluster region
DMCI	Meiosis specific recombinase
NHEJ	non homologous end joining
PAR	poly (ADP) ribose
RAD51	Recombinase enzyme
53BP1 protein	HR suppressor factor
DSS1	Small acidic proteins
OB	Oligosaccharide binding
CHK1	Check point kinase 1
BARD1	BRCA1- associated ring domain
P53	Tumor protein 35
ATM	Ataxia Telangiectasia Mutated
CtIP	Carboxyl –terminal binding protein
DSGs	Daughter strand gaps

BIC:	Breast cancer information core
LGR:	Large genomic rearrangements
ARMS-PCR	Amplification Refractory Mutation System-Polymerase Chain Reaction
SNP	Single nucleotide polymorphism
VUS	Variants of un significance
MLPA	Multiplex ligation probe amplification
DHPLC	Denaturing high performance liquid chromatography
SNV	Single nucleotide variant
RFLP	Restriction fragment length polymorphism
FM	Frameshift mutation
MS	Missense mutation
NS	Nonsense mutation
AA	amino acid
MS-MLPA	Methylation-specific multiplex ligation-dependent probe Amplification
NLSs	Nuclear localization signals
HER2	Human Epidermal growth factor receptor 2

# **Hereditary Breast Cancer caused by germline mutations in BRCA1 and BRCA2 genes in Palestinian women**

## **Abstract**

Breast cancer is the most common malignancy among women worldwide and constitutes 22.9% of all cancers in women. In Palestine, breast cancer is ranked the first among other cancers and constitutes 18.3% from all reported cancers with a mortality rate of 9.8%. Hereditary breast cancer accounts for 5-10% of all breast cancers. Germline mutations in the BRCA1 and BRCA2 genes are responsible for hereditary breast cancer and accounts for 25% of family risk. Other genes are also associated with hereditary breast cancer but to lesser extent than the BRCA genes. Tumors caused by germline mutations in BRCA1 gene have histologically aggressive features and are triple negative. Patients with a family history of breast cancer are advised to make genetic testing to find out if they are carriers of any pathogenic variant and to take preventive measures as annual mammography, MRI, and mastectomy to reduce risk of breast cancer (BC). This study was conducted to screen for the most common pathogenic variants that span different exons in BRCA1 and BRCA2 genes among Palestinian women from Gaza Strip and the West Bank. DNA was extracted and selected regions were amplified by PCR. A total of 50 patients and 50 controls were enrolled in the study. Three different techniques were used to detect these mutations: ARMS-PCR, mismatch PCR-RFLP and PCR-RFLP. The results of the study were as follows: 3 probands had E1250X mutation and one proband had an 185delAG mutation which is considered the second most frequent mutation worldwide after 5382insC mutation.

## سرطان الثدي الوراثي و التوصيف الجزيئي للجينات المرتبطة بها (BRCA1 ,BRCA2) وانتشارها بين النساء الفلسطينيات

### ملخص البحث

يعتبر سرطان الثدي الورم الأكثر شيوعاً بين النساء في العالم بنسبة 22.9% من جميع أنواع السرطان الذي يصيب النساء. يصنف سرطان الثدي في المرتبة الأولى من بين أنواع الأورام المبلغ عنها في فلسطين ويشكل 18.3% بمعدل وفيات 9.8%. الوراثة مسؤولة عن 5-10% من إجمالي حالات سرطان الثدي وتشكل الطفرات الجينية في جينات BRCA1 و BRCA2 25% من مخاطر نشوء الحالات الوراثية. يوجد جينات أخرى لها علاقة بسرطان الثدي الوراثي ولكن بدرجة أقل من جينات BRCA. إذ تكون الأورام الناتجة عن طفرات السلالة الجرثومية في جين BRCA1 أكثر عدوانية لتمكنها من غزو الأنسجة المختلفة في جسم الإنسان بالإضافة إلى كونها سلبية ثلاثية لاستقبال الهرمونات. ينصح المرضى الذين يوجد حالات سابقة في العائلة لسرطان الثدي بإجراء اختبارات جينية لتحديد وجود العوامل المسببة للمرض واتخاذ تدابير وقائية مثل التصوير الشعاعي للثدي، التصوير بالرنين المغناطيسي، واستئصال الثدي للحد من مخاطر الإصابة بسرطان الثدي. الهدف من هذه الدراسة الكشف عن الطفرات الأكثر شيوعاً والتي تمتد عبر exons مختلفة في جينات BRCA1 و BRCA2 بين النساء الفلسطينيات من قطاع غزة والضفة الغربية. تم استخراج الحمض النووي وزيادة أو تضخيم المناطق التي قد تحتوي على الطفرات الوراثية بواسطة PCR. كانت نتائج الدراسة على النحو التالي: 3 طفرات في E1250x، طفرة في 185delAG، الذي يعتبر ثاني طفرة الأكثر شيوعاً في جميع أنحاء العالم بعد طفرة 5382insC.

**Hereditary Breast cancer caused by germline mutations in  
BRCA1 and BRCA2 genes in Palestinian women**

## **Chapter 1**

### **Introduction**

#### **1.1 Background**

Breast cancer (BC) is the most common malignancy worldwide and constitutes 22.9 % of all cancers in women (Armaou et al , 2009). According to the Palestinian Health Annual Reports in 2015, Breast cancer ranked first among other cancers constituting 17.8% from all reported cases with a mortality rate of 11.2%. Nablus and Bethlehem were reported with highest incident rates (Palestinian annual report, 2015). In 2016, breast cancer ranked also the first type of cancer in the West Bank among all type of cancers, where it accounted for 18.3% from all reported cases , with a mortality rate of 9.8% where Bethlehem and Jericho were reported with the highest incidence rates (Palestinian annual report, 2016).

Breast cancer can be invasive when the tumor reaches the fatty and connective tissues and noninvasive if it is confined within the ducts as in ductal carcinoma in situ (DCIS) or in the milk glands as Lobular carcinoma in situ (LCIS) which is less common (Sharma et al .2010).

The majority of breast cancer cases are sporadic and accounts for 70 - 80 % (Goldberg ,Borgen, 2006) ,while hereditary breast cancer accounts for about 5-10 % of all breast cancers , characterized by early age of onset , bilateral breast cancers , ovarian cancers and male breast cancers (Honrado ,Bentez ,Palcaios , 2005) .

Many risk factors (life style and environmental factors) and genetic predisposition increase the risk of developing BC (Sharma et al , 2010) . A family history of breast cancer increases this risk (Beral et al , 2001) .

Germline mutations in BRCA1 and BRCA2 are responsible for hereditary breast cancer and accounts for 25-28 % of the family risk (Gerdes , Cruger , Thomassen , Kruse ,2006 ; Melchor , Benítez , 2013) . Carriers of germline mutations in BRCA1 and BRCA2 genes have a high risk of developing ovarian , fallopian tube cancers , male breast cancer, prostate cancer, pancreas cancer, gastrointestinal cancers and melanoma (Thompson et al , 2004 ; Diez et al, 2003).

The average age of onset for breast cancer is 40 to 50 years for BRCA1 and BRCA2 mutation carriers, and 60 to 70 years for sporadic cases (Petrucci et al , 1993) . BRCA1 mutations are associated with earlier onset age when compared to BRCA 2 mutations (Van Der Kolk et al , 2010).

Tumors caused by mutations in BRCA1 gene are basal or triple negative characterized with lymphocytic infiltration and histologically aggressive features. Tumors associated with mutations in BRCA 2 are luminal, estrogen receptor positive and progesterone receptor positive (Salhab et al, 2010) .

BRCA1 and BRCA2 genes are tumor suppressor genes that has a role in the maintenance of the genome stability by repairing the double strand break (DSB) by homologous recombination (HR) (Roy, Chun , Powell ,2011) . Any alterations or variant in these genes can lead to tumorigenesis.

In this study. We will screen for the mutations that span BRCA1 and BRCA2 genes in Palestinian women.



## 1.2 Hereditary breast cancer

A genetic predisposition of BC increases the risk of BC occurrence in high risk women . A predisposing gene is identified in nearly 30% of cases with family history of BC. About 25% of these cases are due to mutations in rare high penetrant genes (BRCA1, BRCA2, PTEN, TP53, CDH1, and STK11), while 2-3% of the cases are due to mutations in rare low penetrant genes (CHEK2, BRIP1, ATM, and PALB) (Shiovitz et al ,2015) . In a previous study, Wooster et al in 2003 showed different percentage of these genes with associated risk . Figure 1 below shows the genetics of breast cancers.

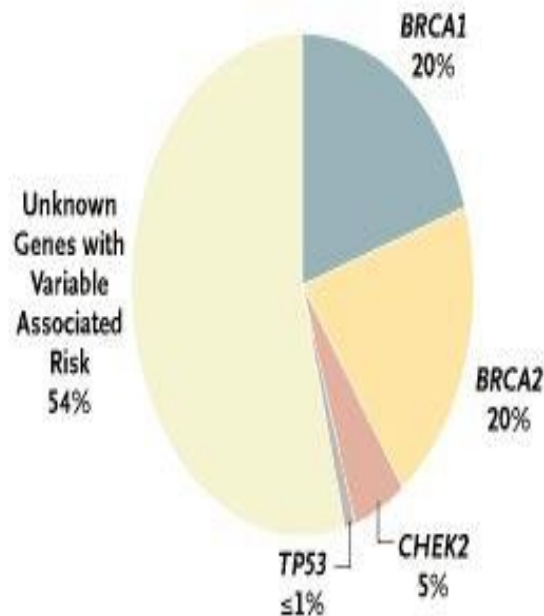


Figure 1: The genetics of breast cancer adapted from Wooster et al, 2003

A patient is considered to have a family history of breast cancer if there is:

- 2 cases of breast cancers in close relatives (1 case diagnosed before 50 years)
- 2 cases of breast cancer cases diagnosed before 40 years
- 3 cases of breast cancers or /and 1 ovarian case in a family
- Early onset of BC
- Ashkenazi Jewish with Breast and ovarian cancer in the same patient (Balmana et al ,2011)

Inheritance of a germline mutation in BRCA genes represents the first hit of Knudson's two-hit model theory of tumorigenesis. Mutations in the BRCA genes affect DNA repair function by homologous recombination resulting in accumulation of aberrant chromosomes, thus predisposing to cancer. If the wild type allele is mutated, this will cause loss of heterozygosity (LOH) and Breast cancer develops (Pasche et al, 2008).

### **1.3 High penetrance genes**

Breast cancer susceptibility gene BRCA1 was first identified in 1990. It is the major gene responsible for hereditary breast cancer. In 1994 BRCA2 gene was identified (Wooster et al ,1994)). BRCA genes are high and rare penetrant genes, they are tumor suppressor genes that are involved in double strand DNA (ds DNA) break repair (Stratton et al, 2008). Any variations or mutations in these genes increase the risk of breast and other cancers mainly hereditary breast and ovarian cancers (HBOC) .Tumors caused by mutations in BRCA1 gene are called triple negative tumors. Tumors have basal like phenotype, high histological grade and do not express estrogen receptor(ER) , progesterone receptor (PR) and HER2 (Rakha et al ,2008). Tumors caused by mutations in BRCA 2 resemble sporadic cancer (Lakhani et al ,2002), have luminal phenotype and expressing ER and PR and the cytokeratins CK8 and CK18 (Lakhani et al ,2002)

Females that are carriers of mutations in either BRCA1 or BRCA2 have a 50-85% life time risk of BC. (King et al, 2003;Lindor et al ,2008). Male carriers have 5-10% life time risk (Liede et al, 2004). Also carriers of BRCA1 mutations have a 10-40% life time risk of ovarian cancer and 10-20% for BRCA2 carriers ( King et al,2003 ;Thompson et al, 2002).

Mutations in BRCA1 and BRCA2 genes are responsible for 15% of hereditary breast cancer (Stratton et al, 2008; Antoniou et al ,2006) . Ashkenazi Jews have higher frequencies due to founder mutations (BRCA1, 185delAG, BRCA1, 5382insC, and BRCA2 6174delT) and these mutations account for 10% of hereditary breast cancer (King et al, 2003).

High and rare penetrance genes other than BRCA1 and BRCA2 are PTEN (Fitz Gerald et al ,1998;Tan et al ,2012) ,TP53 (Garber at al 1991; Rappako et al ,2001; Birch et al ,2001), STK11(Boardman et al ,1998;Lim et al ,2004) and CDH1 (Pharoah et al ,2001). Mutations in these genes have different clinical syndromes. Mutations in TP53 is associated with Li-Fraumeni Syndrome, PTEN is associated in Cowden syndrome and STK11 is associated with Peutz Jeghers syndrome (Turnbull et al, 2008;Tan et al ,2008;Walsh et al ,2007) . High penetrance genes account for nearly 28% of hereditary breast cancer (Antoniou et al ,2006 ;Walsh et al ,2008).

#### **1.4 Low and moderate penetrance genes**

Germline mutations in BRCA 1 and BRCA2 and other high penetrance genes account for nearly 25% of hereditary breast cancer cases, and the remaining variations are due to low and moderate penetrance genes (Lichtenstein et al ,2000). These genes are CHEK 2 (Meijers et al ,2002) ,BRIP1 (BACH1) , ATM(Renwick et al 2006) and PALB2 (Rahman et al ,2007). Studies in the

UK population have estimated that mutations in the moderate penetrance genes accounts for <3% of hereditary BC (Statton et al ,2008) .

Low and moderate penetrance genes are involved in DNA repair and the maintenance of the genome stability, and are associated with cancer syndromes.

CHEK2 is a protein kinase that has a role in cell cycle regulation at G2 phase. In response to DNA damage, CHEK2 is activated and stabilizes P53 and interacts with BRCA1. The most common mutation in CHEK2 is 1100delC .This mutation confers two fold increase in hereditary breast cancer (Shaag et al, 2005).

BRIP1 interacts with the c- terminus domain (BRCT) of the BRCA1 gene . Mutations in BRIP1 (BACH1) accounts for <1% of hereditary breast cancer cases. The mutations are protein truncating and these mutations are associated with Fanconi anemia type j (Seal et al ,2006) .

ATM is a protein kinase that is involved in dsDNA repairing and in regulating CHEK2 and BRCA1. Biallelic mutations in ATM genes causes ataxia telangiectasia (Renwick et al 2006).

BALB2 encodes a protein that interacts with BRCA2 and is involved in nuclear localization.

Biallelic mutations in the BALB 2 genes causes Fanconi anemia type N (Adank et al, 2001).

Mutations in BALB2 accounts for 1-2% of hereditary breast cancer (Rahman et al ,2007,Teo et al ,2013) ,

TGFBR1 gene is a low penetrance gene. TGFBR1\*6A is a common variant in this gene (Pasche et al, 1998). This variant switches the growth stimulatory signals in BC cells (Pasche et al, 1999; Pasche et al 2005).

It was estimated that the population attributable risk (PAR) for breast cancer is 4.9% (Muto et al, 1996). This value is identical to the PAR value for BRCA1 and BRCA2 .Table 1 below shows the breast cancer susceptibility genes

Gene	Associated syndrome	Gene location	Gene frequency	Penetrance
<i>BRCA1</i>	Hereditary breast and ovarian cancer	17q21	Rare	Very high
<i>BRCA2</i>	Hereditary breast and ovarian cancer	13q12.3	Rare	High
<i>TP53</i>	Li-Fraumeni	17p13.1	Very rare	Very high
<i>PTEN</i>	Cowden	10q23.3	Very rare	High
<i>ATM</i>	Ataxia-telangiectasia	11q22-q23	Common	Low to moderate
<i>STK11</i>	Peutz-Jeghers	19p13.3	Very rare	High
<i>TGFBRI*6A</i>	None to date	9q22	Very common	Low to moderate
<i>TGFB1 LI0P</i>	None to date	19q13.1	Very common	Low
<i>CHEK2*1100delC</i>	None to date	22q12.1	Rare	Moderate
<i>CASP8 D302H</i>	None to date	2q33-q34	Common	Low

Table 1: Breast cancer susceptibility genes adapted from Pasche et al, 2008

### 1.5 Clinical significance of BRCA1 and BRCA2 genes

Mutations and rearrangements in the BRCA1 and BRCA2 genes accounts for 20-25% of familial breast cancer. The frequency of these mutations are 0.1-0.5 % of the population worldwide, and the PAR is 3-7% ( Pasche et al ,2008). PAR for Ashkenazi Jews is 15-30%(Warner et al ,1999) , where 2% of Ashkenazi Jews carry a deleterious mutation in BRCA1 and BRCA2 genes , which is 10 folds more common than the general population (Struewing et al ,1997) . 90% of these deleterious mutations are BRCA1 185delAG, BRCA1 5382insC, and BRCA2 6174delT. Ashkenazi Jews are recommended to do a genetic screening test to check if they are carriers of these deleterious mutations to take preventive measures.

It was estimated that a deleterious mutation in the BRCA1 gene confers a 20% of developing BC by the age of 40 .This risk increases with age and reaches an 82% life time risk by age 80 (King et al ,2003).

The risk for ovarian cancer also increases by age for carriers of mutations in the BRCA1 genes, with a life time risk of 54% by the age 80 ( Antoniou et al ,2003).

The available data suggests that BRCA1 penetrance to breast and or ovarian cancer is higher than BRCA2, where carriers of BRCA2 mutations have a lower life time risk (Pasche et al, 2008)

### 1.6 Molecular subtypes of breast cancer

Molecular subtypes of BC is important to determine the most suitable and effective treatment plan.

BC is divided into six main molecular subtypes as shown in Figure 2 below:

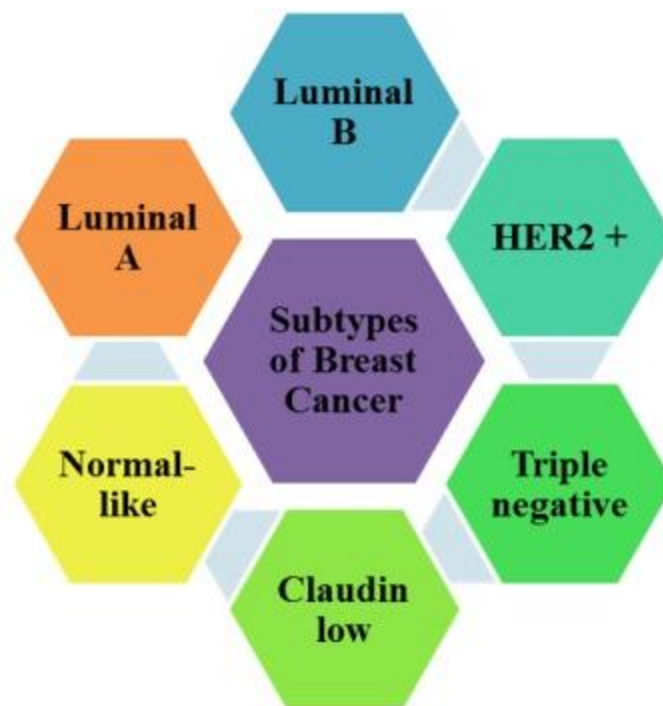


Figure 2: Six main molecular subtypes of breast cancer adapted from Mehrgou et al ,2016

The six main molecular subtypes are Luminal A, Luminal B, triple negative/ basal like, HER-2 type, Claudin –low and normal like.

**1-luminal A:** Luminal cells are breast cancer cells that have phenotypes that resemble the inner lining of the breast ducts. These cells are positive for ER and PR and negative for HER2. Luminal A has best prognosis with high survival rates and are considered to be grade 1 or grade 2 tumors.

**2-Luminal B:** These are positive for ER and or PR and HER2 positive. It has a poor prognosis because of lymph node involvement and large tumor size.

**3-Triple negative /basal like:** Triple negative means that the tumor cells are PR, ER and HER 2 negative. Basal like means that the tumor cells are PR, ER, and Her2 negative and the cancer cells have phenotypes that resemble the outer lining of the breast ducts. Breast cancer that is caused by mutations in BRCA1 genes are triple negative/basal like. This subtype is considered to be aggressive with poor prognosis.

**4-HER2 type:** These tumors are negative for ER and PR. It accounts for 10-15% of breast cancer cases. It has poor prognosis with lymph node involvement and is detected in women at younger age.

**5-Claudin low:** This type is triple negative, with low expression of E- cadherin and lymphocyte infiltration. These features are similar to stem and mesenchymal cells.

**6-Normal like:** These tumors tend to be small with good prognosis, and accounts for 6-10 % of all breast cancer cases (Mehrgou et al ,2016) .

## **1.7 BRCA1 and BRCA2 genes**

### **1.7.1 Structure of BRCA1 and BRCA2 genes**

BRCA1 is located at chromosome 17q21 and it encodes a 1863 amino acids protein. It has a role in the repair of DNA. The coding sequence of the BRCA1 begins in exon 2 and ends in exon 24, where exon 11 encodes 60% of the entire protein (Orban et al, 2003). BRCA2 is located on chromosome 13q12.3 and it encodes a 3418 amino acids protein. In BRCA2 exon 11 encodes 50% of the entire protein (Mehta et al 2004). The coding sequence begins in exon 1 and ends in exon 27. The ALU sequence comprises 41.5% of the BRCA1 gene and 4.8% of other repeat sequences. (Zhang et al, 2010).

BRCA1 have 24 exons and BRCA2 have 27 exons . Exon 11 in both genes are the largest exon and reaches 3.5 Kbp in BRCA1. Exon 11 in BRCA1 gene harbors the most frequent mutations that causes breast cancer.

BRCA1 protein contains a zinc finger in the amino terminal domain , a C3HC4 type ring (ring or zinc) finger. The ring (ring or zinc) finger with 2 zinc atoms and 8 amino acids form a loop like structure .The BRCA1 gene also has a BRCA1 C terminus (BRCT) domain , a nuclear export signal and a nuclear localization signal. Figure 3 below shows the structure of BRCA1 protein structure.





Figure 3: BRCA1 protein structure with different domains adapted from Donovan et al, 2010.

The Zinc finger structure contains a highly conserved Cys3HisCys4 amino acid motif. Any variation in this sequence affects the binding capabilities of the BRCA1 protein.

BRCA2 protein has a BRCT domain that is involved in cell cycle checkpoints and in the response to DNA damage. It has also a region for single strand DNA binding and a PhePP motif which is a region with 26 amino acids, where PhePP binds to meiosis specific recombinase (DMCI). In the C-terminus segment of the BRCA2 protein, there is a domain binding single-stranded DNA. This domain contains 3 oligonucleotide-binding (OB1-3) folds and a helical domain that binds double stranded DNA.

The ovarian cancer cluster region (OCCR) is located in exon 11 in the 3' end of BRCA2 gene. Any mutation in this region will increase the risk of ovarian cancer (Smith et al,1996 ; Murphy et al 2010). Figure 4 below shows the different binding sites of the BRCA2 protein.

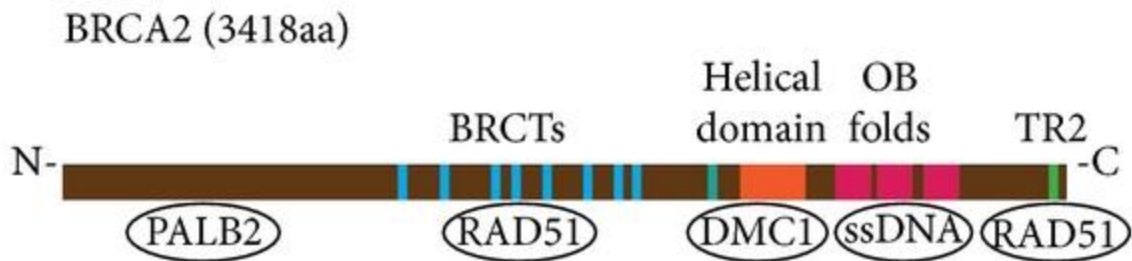


Figure 4: BRCA2 protein structure with different binding sites adapted from Donovan et al , 2010

### 1.7.2 Functions of the BRCA genes

**1-Maintaining genome integrity:** Studies showed that BRCA1 and BRCA2 genes are responsible for maintaining genome integrity during the cell cycle (Scully et al, 2000; Venkitarman et al , 2002) , The precise mechanism is still unclear.

BRCA genes serve as hub proteins that contain disordered regions that become more stable upon binding to other macromolecules (Dunker at al, 2005). Experimental studies showed that the region between residue 170 – 1649 in BRCA genes contains intrinsic disorders (Markel et al ,2005;Pellegrini et al ,2002),

The accumulation of chromosome aberrations during the cell cycle especially in BRCA deficient cells creates double strand breaks (DSBs) or daughter strand gaps (DSGs) during chromosome duplication resulting in stalled forks (Cox et al,2000) . Stalled forks create a DSB that can be repaired by HR , where BRCA genes are involved in efficient HR in mammalian cells (Moynahan et al ,1999;Snouwaert et al ,1999;Moynahan et al 2001;Tutt et al ,2001;Xia et al 2000). In BRCA deficient cells , DNA breaks can be repaired by NHEJ which is error prone mechanism (Patel et al ,1998; Yu et al ,2000 ; Moynahan et al 2001;Tutt et al ,2001;Xia et al 2000).

BALB2 is a partner protein that makes a complex with BRCA1-BRCA2 genes and is involved in localization of BRCA1 and BRCA2 genes to the DNA damage sites (Zhang et al ,2009;Huen et al ,2009). BRCA1 signals the presence of lesions and BRCA2 resolves it by HR with the association of recombinase enzyme RAD51 (Vankitarman, 2014).

The C-terminal of BRCT domains of BRCA1 gene translocate it to the damage sites , where BRCA1 binds poly (ADP) ribose (PAR) chains conjugated to proteins at this site (Yu et al , 2013).

DSBs is repaired by HR , where broken ends must be resected by 5' to 3' nucleolytic activity .

BRCA1 confines HR to the G2 phase of the cell cycle. where a sister chromatid is offered to help in the repair of the damaged site (Bowman et al , 2010; Buntig et al , 2010; Zimmermann et al 2013; Escribano-Diaz et al , 2013). G1 of the cell cycle is inhibited by the HR suppressor factor 53 BPI proteins that bind broken DNA ends and prevents resection. In G2 phase BRCA1 removes 53BP1 and HR starts (Bowman et al, 2010). CtIP attaches to the BRCT domains in BRCA1 (Escribano-Diaz et al, 2013) and regulate the genetic exchange in HR (Chandramouly et al, 2013).

Figure 5 below shows how BRCA genes protect the genome integrity.

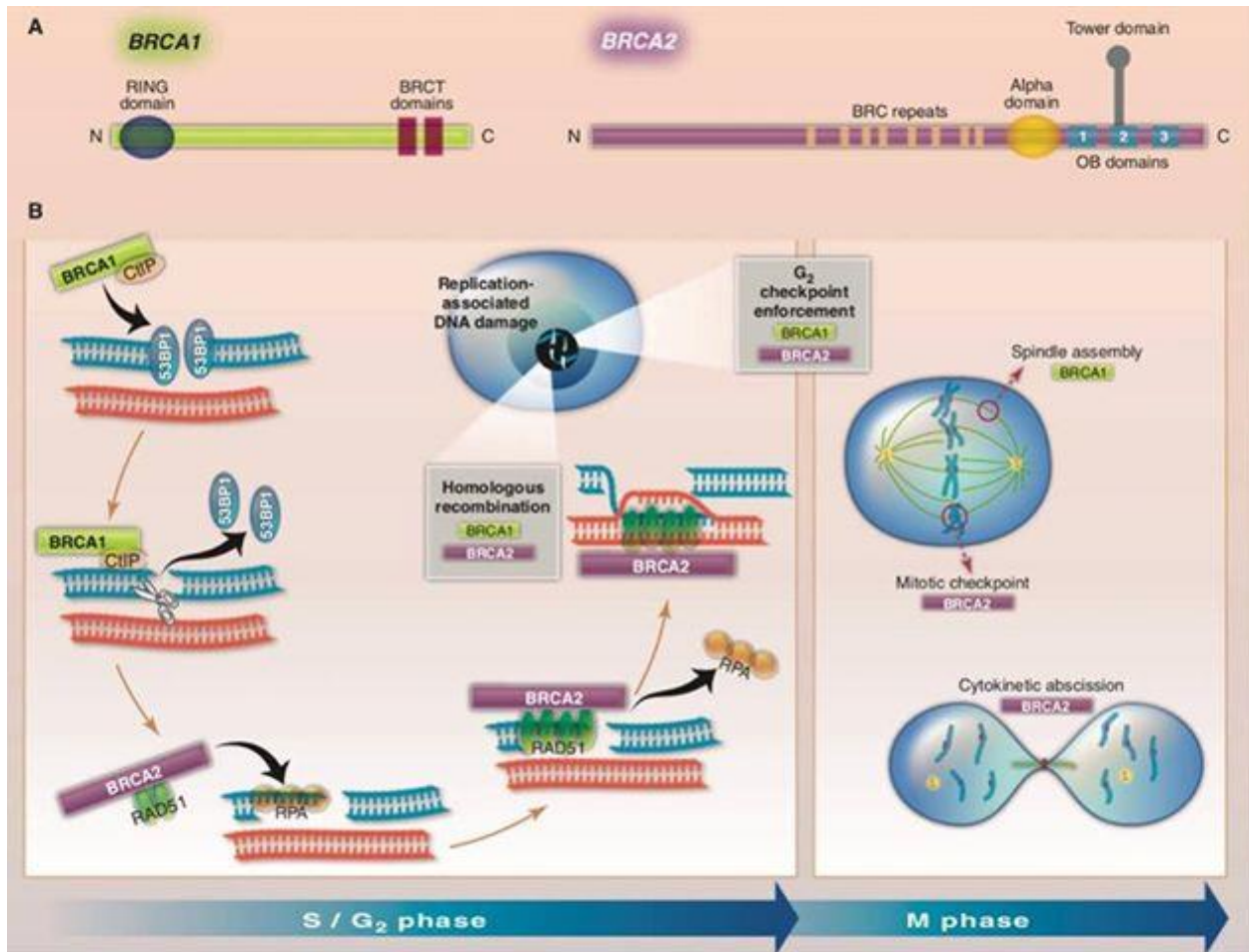


Figure 5: BRCA1 and BRCA2 as chromosome custodians adapted from Vankitarman ,2014

After end resection RPA ss(DNA ) binding protein coats the exposed DNA at DSBs . RPA must be detached to initiate HR. This is attained by a 800 residue segment in BRCA2 gene that binds to DSS1 (small acidic protein) , forming a complex that has an oligonucleotide oligosaccharide binding (OB) folds that are capable of binding to ss(DNA) and ds(DNA ) (Yang et al 2002), This process displaces RPA to allow recruitment of RAD51 (Yang et al ,2005). RAD51 forms a helical filament on ssDNA that mediates interaction with homologous dsDNA in the sister chromatid where strand exchange is essential for repair by HR (Chen et al, 1998) .

**2-Activation of DNA damage checkpoints:** After exposure to genotoxic compounds or radiation, the G2/M cell cycle checkpoints are activated and delay moving the G2 phase into the M phase, thus decreasing the chance of abnormal cells to be passed to the daughter cells. BRCA genes products are involved in the activation of DNA damage checkpoints. Abnormalities in the checkpoints in the G2/M phase in the cell cycle were investigated in murine cells due to disruption of the BRCA1 gene (Xu et al, 1999b). A group of 4 protein kinases are involved in these checkpoints: Tlp1, Chk1p, Rad3p/Mec1p and Cds1p/Rad53p. Each of the checkpoints has ATM, HTR, CHEK1 and CHEK2. These protein kinases phosphorylate and activate BRCA1 gene due to DNA damage after exposure to genotoxic compounds or radiation (Carr.2000).

Mutations in the ATM genes cause ataxia telangiectasia (Shilo.2001), in addition to compromised phosphorylation of the BRCA1 gene predisposing to breast cancer and other kinds of cancers.

CHEK2 kinase is responsible for the phosphorylation of the serine residue ser988 in the BRCA1 gene (Lee et al .2000), and is associated in localization of BRCA1 to DNA damage sites. BRCA2 doesn't have any role in the activation of the cell cycle checkpoints (Patel et al, 1998).

**3-Modulation of chromatin and DNA structure:** BRCA1 has a role in Modulation of chromatin in response to DNA damage through interactions with SWI/SNF proteins that alter chromatin and DNA structure (Bochar et al .2000) and 2 DNA helicases and regulators of histones acetylation and deacetylation (Poa et al ,2000 ; Yarden and Brody ,1999).

The role of BRCA1 in modulation of chromatin and DNA structure is still a subject of investigation.

## **1.8 Mutations and polymorphisms in BRCA1 and BRCA2 genes**

### **1.8.1 Mutations in the BRCA genes:**

BRCA1 and BRCA2 are inherited in an autosomal dominant form and high penetrance in Breast and ovarian cancers (Ayub et al, 2014). The prevalence of the mutations in these two genes is not the same among various ethnic and geographical populations (Neammattzadeh et al, 2015). Inheritance of a germline mutations in the BRCA genes represents the first hit in Knudson's theory, and the second somatic mutations is associated with loss of heterozygosity and deletion of the wild type allele. LOH accounts for more the 60% of tumors from mutation carriers (Zhong et al, 1999; Collins et al, 1995).

Until the year 2014 , 1790 mutations, variants and polymorphisms were found in BRCA1 gene and 2000 mutations were reported in BRCA2 genes according to Breast Cancer Information Core (BIC) database (The Breast Cancer Information Core Database, 2014). Mutations are scattered across the coding regions of the BRCA genes. The most common types of pathogenic variants are nonsense mutations, small deletions or insertions resulting in truncating protein. Large genomic rearrangements and splice sites mutations are also found in the BRCA1 and BRCA2 genes. Silent mutations, polymorphisms and missense mutations are also observed. Nearly 1800 different sequence variants in the BRCA genes are considered to be variants with non-clinical significance (Spearman et al, 2008).

Other studies conducted in 2016 showed that there are 2000 different mutations in the BRCA1 and BRCA2 genes. BRCA1 has higher density of Alu sequences compared to BRCA2 due to higher rates of duplication/deletion in the BRCA1 genes. Large genomic rearrangements accounts for 1/3 of the mutations in the BRCA1 genes that resulted from HR between pseudogenes and BRCA1

gene (Zhang et al ,2010) . Most of the mutations in BRCA2 genes occur in exon 10/11 and include deletions ,insertions leading to promoter stop codon and missense variations leading to a nonfunctional protein (Pisan et al ,2011).Table 2 below shows mutations in BRCA1 and BRCA2 in different countries.

Table 2: Mutations in the BRCA1 and BRCA2 in different countries adapted from Karami et al, 2013

Country	BRCA1 mutations	BRCA2 mutations
Sweden	3172ins5,2594delC 1806C>T,1201del	4486delG
Britain	C4446T,3875del4, 2800delAA,2080delA ,2594delC	6503delTT,9303ins31
Germany	5382insC,4184del4bp, c.3700 3704del5(exon17 and22del Exon13du	3034del4bp,5910C3G, 6676insTA
France	French-Canadian: 4446C>T, 2953delGTAinsC,R1443X, 3875delGTCT French:3600del11, 4184del4,G1710X ,Exons8– 13del Exons3–8and18–20dup	French-Canadian: 6085G>T,8765delAG, 3398delAAAAG, 6503delTT
Italy	c.3228 3229delAG, c.3285delA,c.1377 1378insA c.5062 5064delTGT Exons17,9–19,18–19,1a-2, 16–20del4843delC	1499insA, 7525 7526insT,6174delT c.289G>T,c.2950G>T c.7963C>Tandc.8878C>T
Cyprus	5429delG,3232A.G, 4956A.G	8984delG,1913T>A ,1342C>A,3199A>G, 1093A>C[

Brazil	5382insC	S2219X,C1290Y, 6633del5
Turkey	5382insC,5622C>T	6880insGand3034del AAAC[197
Japan	c.307T>A	5802delAATT,8732C>A, c.2835C>A
Iran	g.4987-68A>G, g.4987-92A>G g.5075-53C>T, g.5152+66G>A g.381 389del9ins29 g.421G>T,g.1286C>T IVS16-92A>G, IVS16-68A>G,4837A>G, IVS18+65G>A, Tyr978X,	c.4415 4418delAGAA c.5529A>C, c.6033 6034insGT c.7242A>G,g.7435+53C>T g.7806-14T>C g.8755-66T>C c.4415-4418delAGAAand c.6033insGT c.5576 5579delTTAA c.9485-1G>A
Lebanon	IVS17-53C>T and g.381- 389del9ins29, 5382insC .G2031T	
India	185delAG, 2983C>A,3450delCAAG ,c.3548A>G, c.- 26G>A,c.317-54C>G , 5341T>G,5364C>G,5379 G>T 1014DelGTand3889DelAG 5382insC	
Nigeria	Exon21del (c.5277+480 5332+672del), intron20(AluSg),intron21 (AluY)	
Egypt	185delAG,5454delC, 4446C>T,738C>A	999del5



Tunisia	330dupA(novel),4160 delAG,the2789delG,5385 insC,c.4041delAG, c.2551delGand c.5266dupC, c.798 799delTT	1537del4and5909insA ,c.211dupA
Algeria	c.46 74del29, c.798 799delTT	
Morocco	c.1016dupA, c.798 799delTT, c.5095C>T,c.4942A>T, c.2805delA/2924delA	c.3381delT/3609delT, c.7110delA/7338delA, c.7235insG/7463insG
Western cape of South Africa	c.1504 1508del	c.2826 2829del, c.6447 6448dup, c.5771 5774del,5999del4
Israel	185delAG,Tyr978X, A1708E,981delAT,C61G	R2336P,IVS2+1G>A 8765delAG

### 1.8.2 Polymorphisms and variants in BRCA1 and BRCA2 genes

Different polymorphisms were observed within the BRCA1/2 genes all over the world. These polymorphisms are harmful and unclassified and their clinical significance is still not clear (Karami et al 2013) .

The most common single nucleotide polymorphisms (SNPs) that were frequently observed in India, Greek ,Maray ,Sri Lanka ,Italy and Turkey is S1613G in exon 16 and P871L/E1038 in exon 11. S1613G is responsible for changing Serine to glycine in Italian women With BRCA1 mutation carriers. (Thommasi et al ,2005; Manguoglu et al ,2010; De Silva et al .2008; Toh et al .2008, Konstantopoulou et al ,2000) . E879E, S919 and Y1137Y were observed in Chinese and Finnish population (Cao et al, 2009).

Another pathogenic variant in BRCA1 is c.1984C.T which changes histidine to tyrosine leading to nonfunctional protein (Loizidou et al, 2007) .

In Czech population , variants that lead to anomalous splicing and truncated proteins are c.302-3c >G in BRCA1 and c.7007 G>G and c.475>A in BRCA2 (Machackova et al .2008) . In German

and Ashkenazi Jews X179C variant in exon 8 in BRCA1 gene was found. This polymorphism has a role in changing the conserved tyrosine (Distelman et al, 2009) .

A Study showed that Y105C, P124H, E143K variants in BRCA1 affect the function of BRCA1 in DNA repair (Wei et al ,2008) , while polymorphisms C5242A in exon 18 of BRCA1 gene results in switching alanine to glutamine and affect the attachment of BRCA1 with proteins involved in DNA repair. These polymorphisms were detected in Hispanic families from western Europe (Torres et al ,2007) .

Healy et al reported that the variant (N372H) in BRCA2 was associated with high BC risk in UK and not associated with high risk of BC in Caucasians , African and Asian populations . Further investigations are needed to confirm this result (Qiu et al , 2010) . The table below summarize the most common polymorphisms around the world.

Gene	Unclassified polymorphism	Missense polymorphisms
BRCA1	IVS7+36T>C,IVS7+38T>C,IVS7+41C>T, IVS7+49del15,(IVS16-68G>A,IVS16-92G>A, IVS18+65G>A S1040N,g.5075-53C>T,g.*381 389del9ins29	E1038G,P871L,K1183R,S1613G,M1652I,D1778G, S1436S,L771L,E1038G, A1708E c.2196G>A,c.3232A>G,c.3667A>G,c.4956A>G , c.5075G>A Q944E,S919P,c.4185+3A>G, c.302-3C>G,c.4185G>Aandc.4675+1G>A, IVS18+1G>T,5632T>A(V1838E), Y179in associationwithF486L,Q356RandS1512I, 3232A>G,L1198WandR1347G,c.9023A/C ,4817A>G[
BRCA2	IVS16-14T>C	c.475G>A,c.7007G>A,c.476-2>G;c.7007G>A; c.8755-1G>A;c.9117+2T>Aandc.9118-2A>G, 1342A>C,S1832P,T2766I,N2781I,andK2860T ,c.72A>T,K3083Eor9475A>G

Table 3: The most common polymorphisms worldwide adapted from Karami et al 2013.

## 1.9 Founder Effect

A founder effect is defined as a common and recurrent mutation that is prevalent in a particular ethnic population or it is the prevalence of the disease in a certain population. Founders are small group of people, where rare mutations become more common within the population as a result of successive interbreeding (Friedman et al, 1995).

Founder populations are important in genetic studies. Founder mutations when detected, make it easier to examine the prevalence of mutations in different ethnicity and the effect of mutation on penetrance and age of onset of the disease (Neuhausen et al, 2000).

The population that has a common and recurrent mutations, genetic testing is considered to be more rapid, easier and less expensive because it is targeted only on the founder mutations (Petrij-Bosch et al, 1997)

One of the most common examples on founder mutations are in Ashkenazi Jews population. Ashkenazi Jews are people that are descendants from central and Eastern Europe. In Ashkenazi Jews , the hereditary predisposition to BC is much higher than other populations because of the founder effect (Roblez-Diaz et al 2004; Antoniou et al ,2003;Liede et al ,2004;Giordano et al .2005;Friedman et al ,1998; Rubinstein et al ,2004), The three founder mutations that are common in Ashkenazi Jews are: 185delAG mutation in BRCA1 gene which accounts to 16-20% of BC diagnosed before 50 (Streung et al ,1995; Brose et al ,2002 ; Roblez-Diaz et al 2004; Antoniou et al ,2003;Liede et al ,2004;Giordano et al .2005;Friedman et al ,1998; Rubinstein et al ,2004; Roa et al ,1996) ,The second founder mutation is 5382insC in BRCA1 gene and accounts for 0.13% ,and the third mutation in BRCA2 6174delT and accounts for 1.52% (Roa et al,1996)

In Iceland the most common founder mutations is 999del5 in BRCA2 gene (Thoralaclaus et al, 1996) with a frequency of 0.4% (Johannesdottir et al. 1996).

Other founder mutations were detected in other populations as in Italians (Cippolini et al,2004), French (Muller et al ,2004), Finns (Sarantaus et al ,2000), Iraqi/Iranians Jews (Quimtana et al ,2005) . Pakistanis (Rashid et al, 2006) and others . Table 4 below shows the most common founder mutations among different populations.

<b>Population</b>	<b>BRCA1 mutations</b>	<b>BRCA2 mutations</b>
Ashkenazi Jews	185delAG , 5832insC	6174delT
Icelanders		6174delT , 995delG
French	3600del11	
Italians	5083del19	
Finns	IVS11 + 3A>G	
Iraqi/Iranian Jews	Tyr978X	
Pakistanis	5454delC , S1503X , R1835X	
Norwegians	1675delA , 816delGT 3347delAG , 1135insA	
Chinese	1081delG	

Table 4 :The most common founder mutations among different populations adapted from Ferla et al ,2007

## Chapter2

### Literature review

The first breast cancer susceptibility gene that predispose to hereditary breast cancer was identified by Miki et al in 1994 .This gene was known as BRCA1. A year later, Wooster at al identified a second gene that predispose to hereditary breast cancer and this gene was BRCA2. Since then, approximately thirty studies have examined the frequency of mutations in these two genes in high-risk breast and ovarian cancer families (Gayther et al, 1998) . After identification of the BRCA1/2 genes (Miki et al, 1994; Wooster at al, 1995) and their localization (Hall et al, 1990), researchers started to study the prevalence of mutations in BRCA1and BRCA2 genes in BC patients and the contribution of mutations in these genes to breast cancer in different populations.

Flora et al in 1998, studied the frequency of common recurrent mutations (185delAG, 5382insC, 6174delT) in BRCA1 and BRCA2 genes in breast cancer patients. DNA was analyzed by allele specific oligonucleotide hybridization . They estimated that the risk of breast cancer in carriers of these three mutations is 36% which is three times the risk of general population.

El-Harith et al, 2002 screened for mutations in major segments of the BRCA1 and BRCA2 genes in Arab and Asian women living in Saudi Arabia . El –Harith and his colleagues used sequencing methods and single strand conformation polymorphism for this purpose. A total of 11 Asian women and 29 Arabian women with breast cancer were recruited in the study .Their findings were as follow: 2482delGACT mutation in the BRCA2 that caused a stop signal at codon 770. The other mutation detected was Arg841Trp in the BRCA1 gene. Six polymorphisms in BRCA2 and five polymorphisms in BRCA1 were also detected . These polymorphisms had different allele frequency.

To study the contribution of mutations in BRCA1/2 genes to breast cancer in Jordanian patients, Abdel –Razzek and his colleagues enrolled one hundred women in the study. All the samples were tested by direct sequencing. The results were as follow: 20 patients had deleterious mutations, 7 patients were suspected to have deleterious mutations in the BRCA1 and BRCA2 genes and 7 patients have variants of unknown significance (VUS) (Abdel –Razzek et al ,2018 ).

Ewald et al in 2009, studied the large rearrangements in the BRCA1 and BRCA2 genes and their frequency among distinct populations, They found that point mutations were the most common

among HBOC patients and rearrangement within the BRCA genes contributed to one third of the BRCA mutations in specific populations . Different PCR methods were applied to validate these results.

Bu et al in 2016 studied the prevalence of BRCA1/2 mutations in Middle Eastern populations. The study included 818 breast cancer patients where samples were tested by Sanger sequencing. Nine different types of deleterious mutations were identified in 28 cases, and seven recurrent mutations were detected and accounted to 92.9% of all cases. Three recurrent BRCA1 mutations c.1140dupG, c.4136-4137delCT and c.5530delC. c.1140dupG and c.4136-4137delCT were considered to be founder mutations and results were confirmed by haplotype analysis.

El Saghir et al in 2015 screened for mutations in BRCA1 and BRCA2 genes in 250 Lebanese women with hereditary breast cancer between 2009-2012 . All the exons in the BRCA1/2 genes were sequenced . Large rearrangements were detected by multiplex ligation probe amplification (MLPA). They found that 14 patients had a deleterious mutations (7 BRCA1 mutations and 7 BRCA2 mutations. Then seven patients with the BRCA1 mutations were diagnosed with triple negative BC, 31 patients had VUS.

Kang et al in 2016 described the analytic validation of next generation sequencing in detecting mutations in the BRCA genes . They deduced that next generation sequencing is a good method for detecting single nucleotide variants , deletions, insertions and large rearrangements with no errors.

Abdein et al in 2017, screened for SNP in Sudanese patients with BC in three regions in the BRCA1 gene . Two regions in exon 11 and one region in exon 2 in BRCA1 gene were amplified by PCR and then sequenced. The results obtained from the study were as follows: a novel SNP V1736D was detected in three patients with an age less than 45 , and 2 missense mutations , rs1799950 were detected in a 27 years patient .

Kadouri et al in 2007 studied the family history of 31 Palestinian women from eastern Jerusalem, 3 with ovarian cancer and 28 with breast cancer. Denaturing high gel liquid chromatography technique (DHPLC) was applied to screen for mutations in BRCA1/2 genes. They found a novel mutation E1373X in BRCA1 gene in exon 12 in a patient with an ovarian cancer.

In Palestine, little is known about mutations in BRCA1/2 genes, and there is no published literature about pathogenic variants among Palestinian women with BC in the West Bank and Gaza Strip. The only literature found has been done by Salahat M in 2011, where 365 women with BC were enrolled in the study. He found a novel mutation E203X in exon 10 and a novel mutations E2229X in exon 11 in BRCA2 gene. In addition, in an unpublished literature in Palestine Dardouk et al , in 2014 found in her study that 5382insC mutation and 6174delT mutation are considered to be founder mutations for Palestinian population.

In this study we checked for the presence of other six additional mutations that were not tested in the above mentioned studies and are considered to be the most common pathogenic variants in world populations . In addition we compared the frequency of these mutations among Palestinians with other populations . The outcome of the study may initiate a strategy for genetic testing in Palestine in BRCA1 and BRCA2 genes for hereditary breast cancer in high risk families . To achieve this we screened for nine mutations among Palestinian women in exons 2,5,11 ,13 and 20 in the BRCA1/2 genes by three different techniques (Allele specific PCR, mismatch RFLP PCR and restriction fragment analyses PCR).

## **2.1 Objectives:**

1. To screen for mutations in different exons in BRCA1 and BRCA 2 genes.
2. To estimate the fequency of these pathogenic variants among Palestinian patients.
3. To investigate the presence of founder mutation specific for Palestinian population.

## **2.2 Significance:**

BRCA1 and BRCA2 Mutations increase the risk for hereditary breast cancer in Palestine . Each population has its common mutations . We also believe that Palestinian population have their specific mutations in the BRCA1 and BRCA2 genes , where the incidence of these mutations varies widely among other population . Recognizing the incidence and the prevelance of BRCA mutations will help in setting a criteria for geneting counselling in members of high risk families in Palestine . Also understanding the genetic basis of familial breast cancer in Palestine is important

in prevention strategies and in clinical management of breast cancer . Women with increased risk of breast cancer can educate themselves to minimize the risk of breast cancer development by monthly self breast exam , mammography and MRI screening .



## **Chapter 3**

### **Materials and Methods**

#### **3.1: Study design**

The study design is a case control study.

#### **3.2: Study population**

Female patients from the west Bank and Gaza strip region (5 patients from Gaza strip and 45 patients from the West Bank) with hereditary breast cancer with an age ranging from 25-70 years old were enrolled in the study. Patients with breast cancer under the age of 40 years old were also recruited in the study. The patients were selected from the chemotherapy and radiology departments in Augusta Victoria Hospital in Jerusalem. Controls enrolled in the study were healthy females that do not have breast cancer or a family history of breast cancer and matched cases with age and gender.

#### **3.3: Patients enrollment criteria**

Patients with the following criterias were enrolled in the study:

- Females diagnosed with breast cancer with a family history
- Breast cancer Patients with age of onset < 40 years old ( no family history of breast cancer)

#### **3.3: Ethical considerations**

The standards of the ethical committee at Birzeit University were fully followed and implemented throughout the study. Patients who voluntarily agreed to participate in the study signed a consent form (Annex1). The importance of the study and the outcomes and expectations were explained. Participants were given enough time to ask questions and think before obtaining their signatures. They were also informed that they have the total right to stop their participation in the study

whenever they want. Privacy and confidentiality were guaranteed and maintained for all participants.

### **3.4: Data collection**

Data were obtained from the patients that were treated in Augusta Victoria Hospital by face to face interview. Questions about the age of diagnosis of breast cancer , the type of treatment , place of residence and whether the patients had a family history of breast cancer were asked and recorded. Patient's records were not available for our use so we couldn't take any other informations about histological grade of tumor. Controls that were recruited in the study matched the patients in gender and age and were selected from Augusta Victoria Hospital , Birzeit University and from Ramallah neighbourhood after their approval to participate in the study and signing a consent form . All the controls had no family history of breast cancer .

### **3.5: Specimen collection**

Blood samples were collected in EDTA anticoagulated tubes (5-7 ml) from 50 patients with hereditary breast cancer and from 50 healthy female controls . Blood samples were obtained using aseptic techniques by lab technicians in Augusta Victoria Hospital. The blood samples were stored at -20° C until analysis.

### **3.6: Materials**

The following materials, equipments and reagents were used:

- EDTA tubes
- Centrifuge
- Plastic racks
- Vortex spinner
- Disposable syringes
- Micropipettes set: 10, 50, 200 and 1000 ul
- Shaking water bath
- 1X (Tris Acetate - EDTA ) TAE buffer
- 2% agarose gel

- Ethidium bromide
- QIAamp DNA mini kit 250

### **3.7: Molecular testing**

#### **3.7.1 DNA extraction:**

DNA was extracted from peripheral blood by using QIAamp DNA mini kit 250. Procedure was done according to manufacturer protocol. The QIAamp DNA mini kit are designed for rapid purification of an average of 6 ug of total DNA from 200 ul of whole human blood. The QIAamp DNA purification procedure is carried out using QIAamp Mini spin columns in a standard micro centrifuge. The first step is lysis of blood cells using proteinase K and lysis buffer provided by the kit. The lysate buffering conditions are adjusted to allow optimal binding of the DNA to the QIAamp membrane before the sample is loaded onto the QIAamp Mini spin column. DNA is adsorbed onto the QIAamp silica membrane during a brief centrifugation step. Salt and pH conditions in the lysate ensure that proteins and other contaminants, which can inhibit PCR and other downstream enzymatic reactions, are not retained on the QIAamp membrane. Then, the DNA bound to the QIAamp membrane is washed in 2 centrifugation steps using 2 different wash buffers, Buffer AW1 and Buffer AW2 provided by the kit. Wash conditions ensure complete removal of any residual contaminants without affecting DNA binding to the membrane. Purified DNA is then eluted from the QIAamp Mini spin column in a concentrated form in AE Buffer also provided by the kit. The purified DNA is then stored at  $-20^{\circ}\text{C}$  until further analysis.

#### **3.7.2 Determination of DNA concentration and purity:**

The determination of the DNA concentration and purity is done by using a spectrophotometer by measuring the absorbance at 260 and 280 nm. The concentration of the DNA is determined by measuring the absorbance at 260 nm. Purity is obtained by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm. Pure DNA has an  $A_{260}/A_{280}$  of 1.2 -1.9. The spectrophotometer readings of samples and controls concentrations and absorbencies are recorded in Table 5 below.

<b>Patients case</b>	<b>Concentration (ng/ul)</b>	<b>Ratio A260/A280</b>	<b>Controls case</b>	<b>Concentration (ng/ul)</b>	<b>Ratio A260/A280</b>
1	26.5	1.41	1	36.6	1.7
2	39	1.5	2	43.3	1.5
3	41.5	1.71	3	22.9	1.6
4	49.2	1.92	4	22	1.7
5	22.2	1.56	5	32	1.5
6	40	1.5	6	25	1.6
7	22.8	1.9	7	20	1.65
8	36.2	1.7	8	22.4	1.64
9	28.2	1.5	9	27	1.7
10	27	1.5	10	32	1.4
11	30	1.6	11	29	1.7
12	24.9	1.6	12	25.4	1.65
13	26.4	1.83	13	41.3	1.73
14	20	1.78	14	58	1.7
15	32.5	1.7	15	32	1.67
16	32.6	1.33	16	23.5	1.6
17	20	1.7	17	26	1.5
18	24.8	1.69	18	39.4	1.79
19	31.5	1.3	19	38.6	1.6
20	22.6	1.4	20	20.4	1.7
21	20	1.7	21	25	1.74
22	23.7	1.8	22	31	1.82
23	31.1	1.75	23	26	1.69
24	37	1.7	24	39	1.77
25	23.5	1.75	25	22	1.8
26	26	1.77	26	34	1.5

27	39.4	1.67	27	20	1.8
28	38.6	1.82	28	33	1.55
29	20.4	1.5	29	21	1.73
30	22	1.63	30	28	1.83
31	21.7	1.46	31	26	1,7
32	32.8	1.26	32	30	1,5
33	22.1	1.89	33	35.1	1,63
34	30.7	1.6	34	26.4	1.8
35	62.3	1.8	35	28	1.68
36	25	1.72	36	31	1,74
37	21.2	1.76	37	37	1.42
38	47.5	1.7	38	25	1.55
39	19.1	1.5	39	40	1.9
40	20.3	1.69	40	35	1.61
41	22.9	1.71	41	22	1.77
42	27.8	1.79	42	20	1.53
43	26.2	1.6	43	23	1.84
44	20.6	1.7	44	29.7	1.66
45	20.3	1.74	45	39	1.38
46	28.7	1.82	46	29	1.76
47	26.2	1.69	47	36	1.48
48	30	1.77	48	23	1.61
49	35.1	1.8	49	36	1.47
50	26.4	1.5	50	21	1.82

Table5: DNA absorbance at 260 and 280 nm and their ratio for cases and controls.

### 3.7.3 Determination of the DNA quality

The integrity and purity of the extracted DNA was evaluated by using 2% agarose gel electrophoresis. The DNA sample extracted from the EDTA blood was loaded with a loading dye

on the agarose gel stained with ethidium bromide and the integrity of the band was visualized under the UV light . Figure 6 below shows the integrity of the DNA in 5 selected samples.

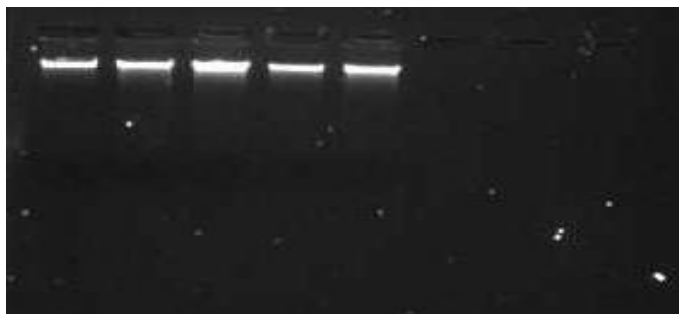


Figure 6: Representative samples for the extracted genomic DNA. The extracted DNA has high quality and not fragmented.

#### **3.7.4 PCR amplification of the BRCA genes:**

The most common and frequent mutations deduced from the database of the BIC were screened . The primers used for PCR have been described in the Breast Cancer Information Core (BIC) database (Annex2) . The forward and reverse primers for each exon and their amplicons size are provided in Table 6 below. The stock primers were dissolved in sterile distilled water to 100Mm. 10ul of each stock primer was then dissolved in 90ul sterile distilled water to obtain a working solution of a final concentration of 10uM . PCR amplification were carried out in a reaction volume of 25 ul containing 12.5 ul of Ready Master mix (Thermo Fischer Scientific ) , 10 ul of distilled water . 0.5 ul of forward and reverse primers each and 1.5 ul of DNA template. The DNA amplification was performed in the thermal cycler using the following conditions: complete denaturation (98°C for 5 min), followed by 35 cycles of amplification (98°C for 0.4 min, 55°C for 0.5 min and 72°C for 0.5 min) and the final extension step (72°C for 7min). The PCR product was electrophoresed on 2% agarose gel prepared with 1X TAE buffer and ethidium bromide and visualized under UV light using the Gel Doc XR system .

Mutation	Exon	Primer Sequence (5' to 3')	Type of mutation	AA change	Base change
185delAG	2	F:AAA ATG AAG TTG TCA TTT TAT AAA CC R:CTG ACT TAC CAG ATG GGA CAT T	FS	Stop 39	delAG
5382insC	20	F:CCA AAG CGA GCA AGA GAA TC <u>A</u> C R:GAC GGG AAT CCA AAT TAC ACA G	FS	Stop 1829	insC
E1250X	11	F:GAT GAC CTG TTA GAT GAT GGT GA R:CTC TGT GTT CTT AGA CAG ACC <u>C</u> T	NS	Gln to Stop	G to T
C61G	5	F:CTCTTAAGGGCAGTTGTGAG R:ATGGTTTTATAGGAACGCTATG	MS	Cys to Gly	T to G
1675delA	11	F:TTCATCCTGAGGATTTTATCTA R:CATGAGTTGTAGGTTTCTGCTG	FS	Stop 531	delA
Q563X	11	F:ATGATAAATCAGGGAACTAACC R:CATGAGTTGTAGGTTTCTGCTG	NS	Gln to Stop	C to T
Q780X	11	F:CACCTAAAAGAATAGGCTGAG R:AGTAATGAGTCCAGTTTCGTTG	NS	Gln to Stop	C to T
R1443X	13	TCT TCT GCC CTT GAG GAC <u>A</u> TG GGG ACA AGA ACC AAG GCT CC	NS	Arg to Stop	C to T
6174delT	11	CF- AGCTGGTCTGAATGTTTCGTTACT RP1=GTGGGATTTTTAGCACAGCTAGT RP2- CAGTCTCATCTGCAAATACTTCAGGGATTTT TAGCACAGCATGG	FS	Stop2003	del T

Table 6: Mutations, 5' to 3 primer sequence, type of mutation and amplicon size adapted from Meindl et al, 2002

### 3.7.5 Amplification Refractory Mutation System-Polymerase Chain Reaction ARMS- PCR:

ARMS -PCR was used to detect 6147delT mutation in exon 11 in BRCA2 gene in the collected samples. For the detection of this mutation, specific oligonucleotide primers that are

complimentary to the mutant type sequence or the wild type sequence were used (Sirisha et al, 2017). The primers used for the detection of the mutation 6147delT were as follow: Common forward primer (CF) , reverse wild type primer (RP1) and reverse mutant type primer (RP2) . A 151 base pair region for wild type and 171 base pair region for mutant type were amplified. The reaction took place in a 25 ul volume consisting of 12.5 ul of Ready Master mix (Thermofischer scientific), 9.2 ul distilled water , 0.4 ul each of reverse and forward primers and 2.5 ul of DNA template. The forward primer was the common primer that was used with the reverse wild type primer in the first reaction and the reverse mutant type primer in the second reaction . The thermocycler conditions for DNA amplification were as follows : complete denaturation (98°C for 5 min), followed by 35 cycles of amplification (98°C for 0.4 min, 55°C for 0.5 min and 72°C for 0.5 min) and the final extension step (72°C for 7min) . The amplicons were migrated on 2% agarose gel stained with ethidium bromide. Gels were visualized under UV light .

### **3.7.6 Mismatch PCR –RFLP:**

Mismatch PCR RFLP technique was used to detect single nucleotide polymorphism (SNP) that doesn't affect any restriction sequence. The primers used in mismatch PCR RFLP contains also mismatch bases near the restriction site (Halliassos et al, 1989). In mismatch PCR RFLP the reaction was held in a 25 ul volume that consists of 12.5 ul of Ready Master mix , 10 ul of distilled water , 0.5 ul of each forward and reverse primers and 1.5 ul of DNA template. The PCR protocol was as follows: : complete denaturation (98°C for 5 min), followed by 35 cycles of amplification (98°C for 0.4 min, 55°C for 0.5 min and 72°C for 0.5 min) and the final extension step (72°C for 7min). The PCR product was visualized with 2% agarose gel prepared with 1X TAE buffer and ethidium bromide. Nested PCR was recommended because the primer could not amplify the DNA



fragment because the mismatch was designed on the last two nucleotides of the primer. The nested PCR was held in a 25 ul volume as the conventional PCR and the only difference is that 1.5 ul of the PCR product is added instead of the DNA template. The PCR protocol was the same as the conventional PCR . Restriction endonuclease has been used for enzymatic digestion of the restriction site. The restriction protocol was as follows: 2 ul of Restriction enzyme (Biolabs) , 3 ul of 10x buffer R , 15 ul of distilled water (up to 30 ul) and 10 ul of PCR product . The reaction tubes were then incubated at 37° C overnight as recommended by the manufacturer instructions. The digested fragments were visualized on 2% agarose gel electrophoresis stained with ethidium bromide . Table 6 below shows the primers designed to amplify the target sequence, the restriction enzyme used for digestion and the fragment sizes after restriction enzyme assay .

<b>Mutation</b>	<b>5' to 3' sequence</b>	<b>Restriction enz.</b>	<b>Size(Normal)</b>	<b>Size(Mutant)</b>
<i>158delAG</i>	F:GAAGTTGTCATTTTATAAACCTTT R:TGACTTACCAGATGGGAGAC	HinfI	150-20 bp	168bp
<i>1675delA</i>	F:TTCATCCTGAGGATTTTATCTA R:CATGAGTTGTAGGTTTCTGCTG	XbaI	454-19 bp	473 bp
<i>E1250X</i>	F:AGGCATAGCACCGTTGCTTC R:TCTCCAATTCACCTGCACTG	Hpy188 I	167-21 bp	188 bp
<i>R1443X</i>	F:TTCTGCCCTTGAGGACCCG R:ATGTTGGAGCTAGGTCCTTAC	BstUI	123-19 bp	142 bp
<i>5382ins C</i>	F:CCAAAGCGAGCAAGAGAATCTC R:GGGAATCCAAATTACACAGC	DdeI	214-36-20 bp	234-36-20 bp

Table 7: Mutations, primer sequence, restriction enzyme used and size of the fragments after restriction enzyme assay in mismatch RFLP PCR.

### 3.7.7 PCR- RFLP:

RFLP was used to detect the following mutations as shown in table 7 below:

<i>Mutation</i>	<b>5' to 3' sequence</b>	<b>Restriction enzyme</b>	<b>Size(Normal)</b>	<b>Size(Mutant)</b>
<i>C61G</i>	F:CTCTTAAGGGCAGTTGTGAG R:ATGGTTTTATAGGAACGCTATG	HpyCH4 III	154-74-50bp	228-50 bp
<i>Q563X</i>	F:ATGATAAATCAGGGAACCTAACC R:CATGAGTTGTAGGTTTCTGCTG	Hpy188 I	320-98 bp	418bp
<i>Q780X</i>	F:CACCTAAAAGAATAGGCTGAG R:AGTAATGAGTCCAGTTTCGTTG	Hpy188 III	525-380- 183bp	708-380 bp

Table8: Mutations, primer sequence, restriction enzyme used and Size of the fragments after restriction enzyme assay in RFLP PCR

The reaction was held in a 25 ul volume that consists of 12.5 ul of ready master mix, 10 ul of distilled water, 0.5 ul of forward and reverse primers and 1.5 ul of DNA template. The PCR protocol was as follows: complete denaturation (98°C for 5 min), followed by 35 cycles of amplification (98°C for 0.4 min, 53°C for 0.5 min and 72°C for 0.5 min) and the final extension step (72°C for 7min). The PCR product was visualized with 2% agarose gel. The amplified gene was then digested with restriction digestion with the following protocol: 10 µl of amplified product, 3 µl 10x buffer, 1.5 µl restriction enzyme and 15.5 µl H<sub>2</sub>O (up to 30 µl total). Then the reaction tubes were incubated at 37°C for 2 hours as indicated in the manufacturer technical bulletin. The sample is then run on a 2 % agarose gel.

## **Chapter 4**

### **Results**

We have studied 50 patients with hereditary breast cancer with mean age of 36.3 years and 50 healthy controls with a mean age of 37.6 years old. We screened the samples for the most common mutations in different exons in the BRCA1 and BRCA2 genes (2,5, 11 ,13,20) according to the BIC database which is considered a reliable online mutation database for breast cancer susceptibility genes . Three different techniques were used in this study : ARMS-PCR, mismatch RFLP and restriction fragment analysis.

#### **4.1: BRCA2 mutations**

##### **A: 6174delT mutation**

Allele specific PCR was used to detect the 6174delT mutation in exon 11 in BRCA2 gene. The 50 patients and healthy control were homozygous for the wild type allele and all were negative for this mutation. Amplification with the wild type specific primers gave a 151 bp band in the wells in lane 1 which represents normal exon sequence size and the amplification with the mutant type primers gave no bands as seen in lane 2 in figure 7 below which is supposed to give a 171 bp band in mutant cases .

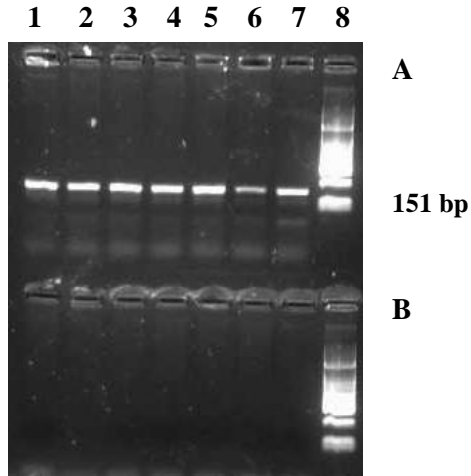


Figure7 A: lane 1-7 amplification of the wild type allele in exon 11 in BRCA2 and a 151 bp segment was shown indicating homozygous wild type allele for the 617delT mutation. Lane 8 is 100 bp ladder. 7B lane 1-7 represents the absence of the amplification of the mutant type allele indicating the absence of 6174delT mutation.

## 4.2 BRCA1 mutations detected by mismatch RFLP-PCR technique

### 4.2.1 185delAG mutation

Restriction result of BRCA1 185delAG using HinfI enzyme as shown in Figure 8 below showed that well number 1 gave a band of 168 bp which means that this sample was not restricted by the HinfI enzyme and so sample 1 was positive for this mutation. The frequency of this mutation was 2%. Samples 2-6 gave a 150-20 bp bands which is the normal exon band size obtained from the restriction of the enzyme to the DNA fragments. All the other 49 samples and 50 control were negative for this mutation.

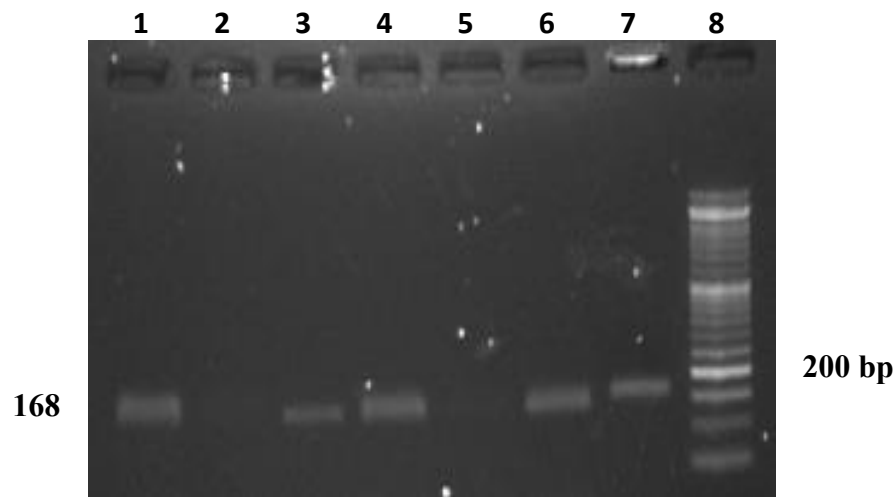


Figure8: Restriction of PCR-RFLP BRCA1 amplicons for the detection of 185delAG after treatment with HinfI enzyme, , well 1 is positive for the restriction (168 bp fragment). Wells 2-6 gave normal restriction of 2 fragments of 150 and 20 bps. Well 7 is a control (pcr product ) and well 8 is a 50 bp ladder.

#### 4.2.2 1675delA

All 50 patients and controls were negative for this mutation as shown below in Figure 9. All samples were restricted by Xba1 enzyme and a normal exon band size were obtained in all samples which is 454 and 19 bp. There were no samples with a 473 bp fragment which means that all samples were negative for this mutation. Figure 9 below shows restriction results of 1675delA by Xba1 enzyme

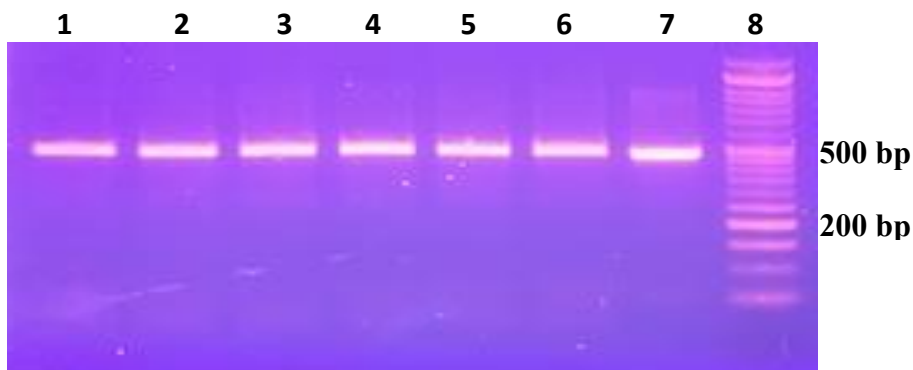


Figure 9 : Restriction result of PCR-RFLP BRCA1 for the detection of 1675delA mutation after digestion by Xba1 enzyme . Wells 1-7 represent normal samples of 454 and 19 bps. Well 8 is 50 bp ladder.

### 4.2.3 E1250X mutation:

Restriction result by the enzyme HPY1881 showed as seen in Figure 10 that 3 samples in wells 1,2,4 were not restricted by the enzyme and gave a band of 188bp which is an indication of the presence of E1250X mutation in these samples with a frequency of 6%. Samples that gave 2 bands of 167-21 bp size were negative for the presence of this mutation.

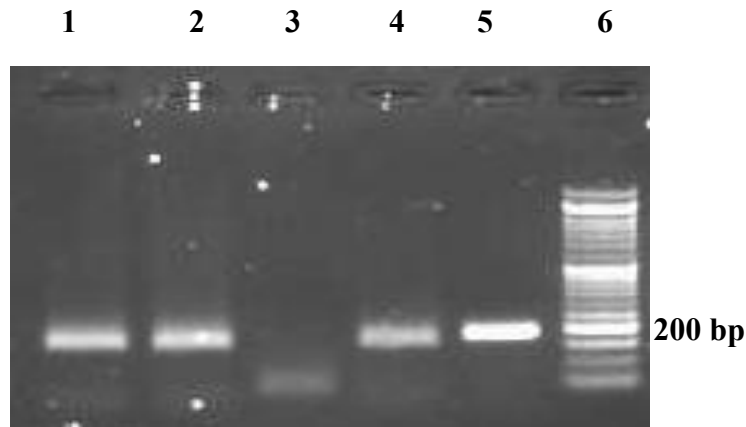


Figure 10: Restriction result of PCR-RFLP BRCA1 for the detection of E1250X mutation after digestion by HPY1881enzyme. Wells 1,2 and 4 are mutated samples and gave a 188 bp fragment. Well 3 represents a normal sample restricted to 2 fragments 167 and 21 bp. Well 5 is PCR amplicon not treated with the restriction enzyme. Well 6 is 50 bp ladder.

### 4.2.4 R1443X mutation

The R1443X restriction results were all negative in patients and healthy controls. The restriction results by the BstU1 enzyme gave 2 bands of 123-19 bp size in all the samples which is the normal exon sequence size as shown in Figure 11 below. No 142 bp band was found, which indicates that all samples were negative for this mutation.

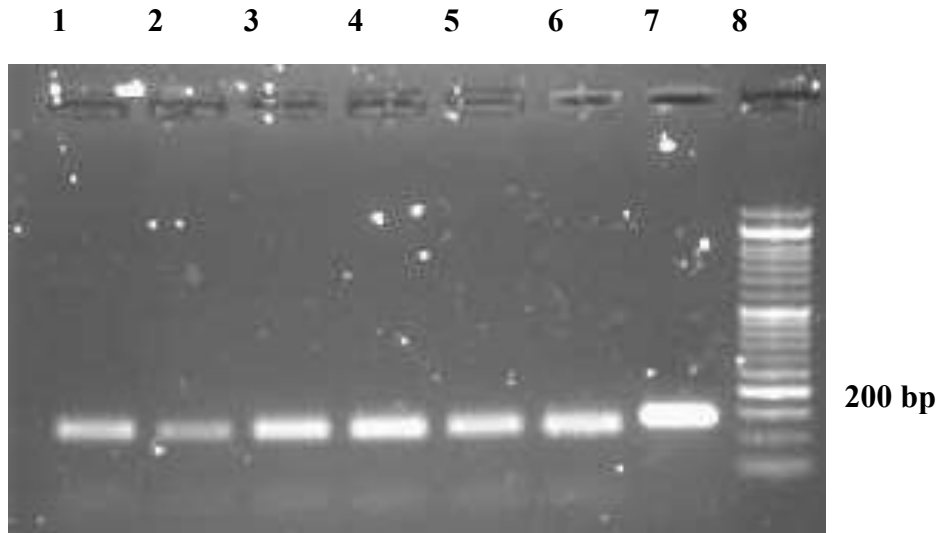


Figure 11 : Restriction result of PCR-RFLP BRCA1 R1443X after digestion by BstU1 enzyme. Well 1-6 are patient samples, all restricted to two fragments of 123 and 19 base pairs. Well 7 is a control and well 8 is a 50 bp ladder.

#### 4.2.5 5382insC mutation

Restriction fragment analysis results for 5382insC mutation gave 3 bands of 214-36-20 bp upon restriction by Ddel enzyme .These sizes are the normal exon sequence size. All samples and controls were negative for 5382insC mutation. Samples that gave 3 bands of 243-36-20 bp are considered to be mutated, but none of the samples tested were positive for this mutation. Figure 12 below shows the results of the restriction of the 5382insC mutation by Ddel enzyme.

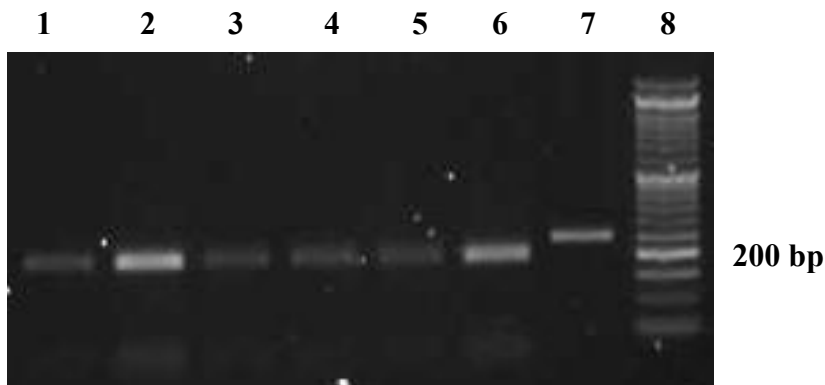


Figure12: Restriction result of PCR-RFLP BRCA1 5382insC after digestion by Ddel enzyme. Well 1-6 are patient samples restricted to 3 fragments 214, 36, 20 base pairs. Well 7 is a control (unrestricted amplicon) and well 8 is a 50 bp ladder

### 4.3 BRCA1 mutations detected by RFLP:

#### 4.3.1 C61G mutation

The restriction enzyme HpyCH4 III was used for detection of C61G mutation, All patients and healthy controls were negative for this mutation and the restriction result showed 3 bands 154-74 and 50 bp as shown in Figure 13 below which reveals that C61G mutation did not exist in tested patients. Samples that gave 2 bands of 228-50 bp are considered to be mutated but none of the samples were positive for C61G mutation.

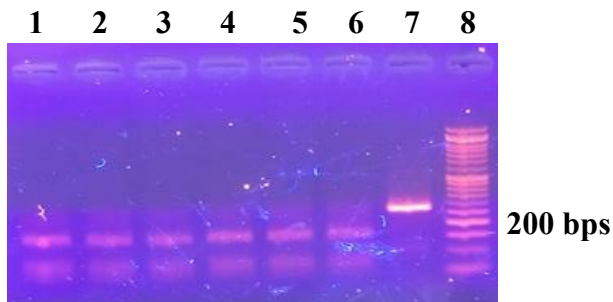


Figure 13: Restriction result of PCR-RFLP BRCA1 C61G after digestion by HpyCH4 III enzyme. Well 1-6 are patient samples restricted to 3 fragments of 154, 74 and 50 base pairs (not mutated). Well 7 is a control (un restricted amplicon), and well 8 is a 50 bp ladder.

#### 4.3.2: Q563X mutation

Q563X mutation was not found in all the patients and healthy controls . Restriction results on the 2% agarose gel by Hpy188 I enzyme showed 2 bands of 320 -98 bp in all of the samples which indicates negative results as shown in figure 14 below .



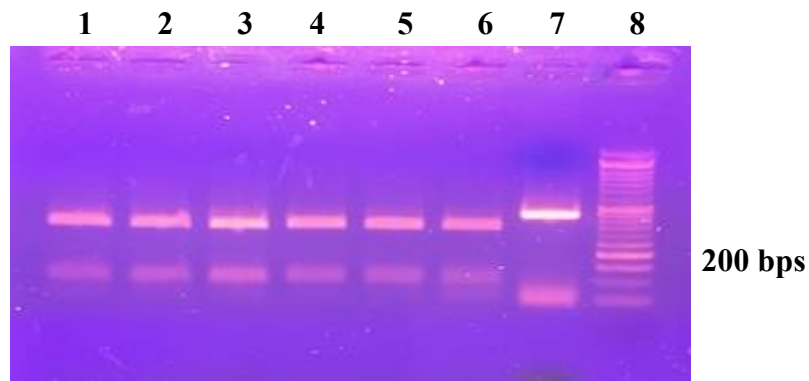


Figure 14: Restriction result of PCR-RFLP BRCA1 Q563X after digestion by Hpy188I enzyme. Well 1-6 are patient samples restricted giving fragments of 320, and 98 base pairs (not mutated). Well 7 is a control (un restricted amplicon), and well 8 is a 50 bp ladder.

#### 4.3.3 Q780X mutation

Restriction by Hpy188 III enzyme gave 3 bands 525-380-183bp .This means that all the samples and controls were negative for this mutation as shown below in Figure 15.

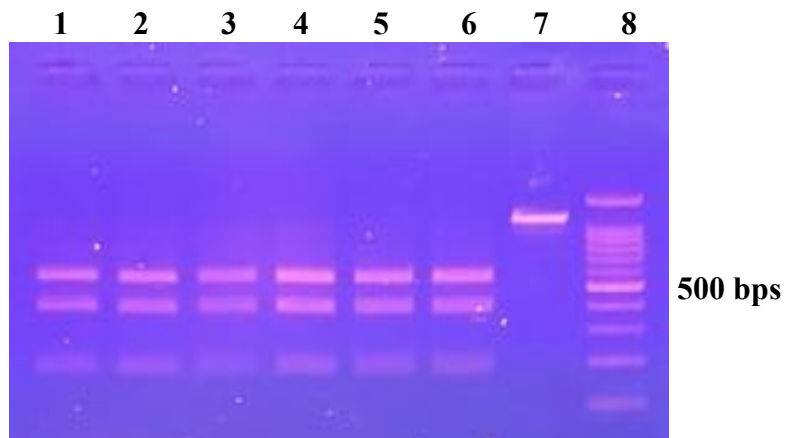


Figure 15: Restriction result of PCR-RFLP BRCA1 Q780X after digestion by Hpy188 III enzyme. Well 1-6 are patient samples that are not mutated (normal) restricted in 3 fragments of 525, 380 and 183 base pairs. Well 7 is a control (un restricted amplicon) and well 8 is a 100 bp ladder.

## Chapter 5

### Discussion

Germline mutations in BRCA1 and BRCA2 genes accounts for nearly 15% of hereditary breast cancer. According to the Palestinian Health annual reports in 2015 and 2016 , we noticed an increase in the number of breast cancer cases and a decrease in the mortality rate which may be due to better awareness among Palestinian patients in making annual mammographies and early diagnosis of breast cancer occurrence . It also may be due to better clinical care applied in the Palestinian hospitals (Palestinian Annual Health Reports 2015 , 2016 ) . The prevalence of BRCA1/2 germline pathogenic variants differs between different ethnic groups (Balmana et al , 2011). The inclusion criteria for candidates for genetic testing is based on specific guidelines. It was estimated based on a clinical criteria that includes family history and age of onset of breast cancer that the pathogenic mutations in BRCA1/2 are identified in 12-15% of tested cases (Balman et al ,2011) . The most common mutations worldwide according to the Breast cancer information core (BIC) database were studied in Palestinian population with hereditary breast cancer. In this study we analyzed 9 most common mutations that is found in exons 2 , 5 ,11 ,13 and 20 in BRCA1 and BRCA2 genes. BRCA1 mutations were found in only 4 patients, 1 patient with 185delAG, age of onset 40 years from Hebron and 3 patients with E1250X mutation with age of onset 43, 70, and 34. The other BRCA1 mutations tested were negative in all patients . 6174delT mutation in BRACA2 gene was also not found in the probands tested.

The 185delAG mutation that was detected in 1 proband has a percentage rate of 2% . The mutation was less frequent in Palestinian population because each population has its own recurrent mutations that differs from other population and the pathogenic mutations varies in different geographical areas. This mutation is a frameshift mutation that causes a premature stop codon at

nucleotide 39 . In other study in Palestine, Dardouk et al, 2014 found that this mutation was negative for all the probands tested . The 185delAG mutation was detected with a high frequency in Ashkenazi Jews (Friedman et al., 1995; Struewing et al, 1995). Other studies have detected the 185delAG mutation in non-Jewish populations (Bar-Sade et al., 1998), including Spanish populations (Osorio et al., 1998; Díez et al., 1998; Díez et al., 1999) and in Chilean population with a frequency of 0.26% (Jara et al., (2002).

In Ashkenazi Jews population, it was estimated that 185delAG mutation contributed to 16% of breast cancer diagnosed at age less than 50 years (Struewing et al. 1995), 20% of the cases were diagnosed at the age less than 40 years (FitzGerald et al. 1996) and 22% were diagnosed at age less than 42 years (Offit et al. 1996). 185delAG mutation is considered to be the second globally frequent BRCA1 mutation and was described in all the ethnics including America ,Africa ,Asia, and European populations (Karami et al ,2013).

The second mutation detected in Palestinian probands was E1250 X with a percentage rate of 6%. This mutation is a deleterious nonsense pathogenic variant that results from a change from G to T at nucleotide 3867 and changes a Glu to a stop codon leading to truncated protein. (Ford et al .1994). This mutation was found in the proband of early-onset breast cancer (age 34) in Indian population (Valarmathi, et al, 2003). From few unpublished literature in palestine , E1250X mutation was not previously screened for its presence among Palestinian population and this is the first study to detect this mutation among the Palestinian population.

The other seven mutations tested were all negative in this study.The mutation 5382insC was negative in all the 50 samples and 50 controls, although this mutation is the most frequent BRCA1 gene mutation which was found in almost all of the populations. This mutation existed at first in Scandinavia probably Denmark. It comprises the founder mutation in Danish population. This

mutation also exists in Russian population which was then disseminated to Ashkenazi Jews. This mutation is the most frequent mutation in European countries (Hamel et al, 2011).

In an unpublished study in Palestine, Dardouk et al, 2014 found in her study that 5382insC mutation was found among Palestinian population with an incident rate of 59.4% and they considered that this mutation was a founder mutation for the Palestinian population where this mutation was negative in all the samples tested in this study. Different techniques were used in detecting this mutation , we applied mismatch RFLP PCR and Dardouk applied allele specific PCR Mismatch PCR is a flexible technique where nearly small deletions, insertions and single-base changes can be identified (Rohlf et al, 1997) .Haplotype analyses was not done to investigate if this mutation is considered to be a founder mutation for Palestinian population. Also the results were not confirmed by sequencing which is considered the gold standard in genetic testing.

6174delT mutation causes a premature stop codon in exon 11 of BRCA2. It occurs in C-terminal of protein and causes disruption of nuclear localization signals (NLSs) which disrupt with BRCA2 role in DNA repair process (Pisano et al, 2011). This pathogenic variation has a role in drug resistance especially PARP inhibitors (Montagna et al, 2003). It is prevalent in Italian population (Karami et al, 2013) and in Ashkenazi Jews (Flora et al ,1998). In my study 6174delT mutation did not exist, where in other studies in palestine by Dardouk et al , 2014 , 6174 delT mutation was considered a founder mutation among Palestinian population with an incident rate of 30%. The method used in detection for this mutation was the same method used by Dardouk et al , 2014 which is allele specific PCR . Sequencing must be done to confirm the results .

C61G mutation was not found in my study . It is a significant mutation in Poland (Jakubowska et al., 2001, van Der Looij et al., 2000a), It is considered to be the second most frequent mutation in the polish population. Its incident rate is very high in Poland compared to other populations

(Goński et al, 2000). This mutation also has been reported in Hungary (van Der Looij et al., 2000b), Germany (Spitzer et al., 2000), and in other regions of Europe (BIC).

The most frequent mutations of BRCA1 gene is 1675 del A. This mutation was negative among the probands tested in my study. 1675delA mutation is reported to be a founder mutation in Norwegian population (Dorum et al 1999. Heimdal et al, 2003).

Q563X, Q780X and R1443X are all point mutations that gives a truncated protein . Q563X and Q780X are nonsense mutations . R1443X is a missense mutation . The three mutations were not found among Palestinian patients tested in this study. These three mutations causes a base change from C to T leading to a stop codon (Jara et al, 2004).

Q563 X,Q780X , R1443X ,1675delA, 6174delT ,5382insC , and C61G mutations were not found in our study because I believe that palestinian population has their own mutations as other populations do and these mutations are not prevelant in Palestinians. Also this may be due that the mutations existed in BRCA1/2 gene was not always due to small insertions or deletions . Recently all the studies regarding the mutations in the BRCA genes are employed to silencing of the BRCA gene as a result to methylation of the cytosine phosphate guanine CpG sites across the promoters of these genes. Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) assay was used to analyze germline BRCA promotor hyper methylation in in breast cancer patients (Vos et al, 2017). Balada et al, 2018 in a recent study stated that there is no hyper methylation in the promoters of the BRCA genes in hereditary breast cancer. More studies need to be done to reject or accept this theory.

Sequencing is considered the gold standard in detecting the pathogenic variants that span the BRCA1/2 genes, but because of deficiency of financial and lab resources, other cost effective

techniques as ARMS, mismatch RFLP and restriction fragment analysis were used and these techniques is considered to have high specificity and sensitivity .

Mismatch PCR technique was used to detect 5 mutations ( 5382insC ,R1443X,1675delA,E1250X ,185delAG ) . This technique is used to detect SNPs that do not affect any restriction sequences (Haliassos et al ,1989) . It is also capable of detecting small insertions, nonsense, missense and deletions by introducing or removing a restriction site by mismatching a base near the mutation in the PCR primers generating a PCR product with the base change. As a result the mutant allele does not have the restriction site that the normal allele has and it is not restricted by the restriction enzyme leading to a larger fragment on the resolution gel compared to the wild type allele. Mismatch PCR is a flexible technique where nearly small deletions, insertions and single-base changes can be identified (Rohlf's et al, 1997). Disadvantages of this technique was that the reverse primer was not able to anneal thus preventing amplification, where different optimization protocols were tested to ensure the amplification of the alleles .

## Chapter 6

### Conclusions and recommendations

In conclusion Breast cancer (BC) is the most common malignancy worldwide and mutations in the BRCA1 and BRCA2 genes contribute to the pathogenesis of hereditary breast cancer. In our study 3 probands had E1250x mutation and one proband had an 185delAG mutation which is considered the second most frequent mutation worldwide after 5382insC mutation. The percentage rate of these mutations are 6% and 2% respectively.

Hereditary breast cancer is caused by high penetrance genes other than BRCA1/2 genes that has a role in DNA repair mechanism and contributes to a significant percentage of hereditary breast cancer and must not be neglected. More studies regarding this subject is required for better and efficient genetic counselling in Palestine.

BRCA1 and BRCA2 are large genes, and complete analysis of the coding regions is expensive and time-consuming. This study is the first step in implementing a strategy for genetic testing in patients with high risk family of breast cancer. The identification of the most common mutations among Palestinians will allow for the rapid diagnosis of BRCA1/2 mutation carriers where the diagnosis and treatment of BC patients and the process of follow-up of their family get faster and better.

The primary goal of genetic screening for pathogenic mutations in BRCA1/2 genes is the identification of cases at the greatest risk of developing breast and ovarian cancer in Palestine and to implement preventative strategies that have a role in patient care and in the identification of patients who are most likely to benefit from emerging therapies targeting DNA repair mechanisms such as PARP inhibitors. There is also a need to broaden the BRCA1/2 testing

criteria in order to optimize the use of BRCA testing to guide treatment decisions by introducing individual criteria, such as including women with triple-negative breast cancer and women with high-grade ovarian cancer (Dominique et al ,2016) .

Complete sequencing of the BRCA genes is recommended to give a better picture of the involvement of BRCA1 and BRCA2 in the incident of breast cancer among Palestinian population. Haplotype analysis is also recommended to provide the complete ensemble of founder alleles existing in the Palestinian population

People in high risk families of hereditary breast cancer must check if they are carriers of the most recurrent mutations among the Palestinian population and take preventive measures as mastectomy ,mammography and other procedures to decrease the risk of breast cancer occurrence.



## Chapter 7

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## Annex 1 : Consent form



المخبرية

برنامج الماجستير في العلوم الطبية

موافقة على المشاركة في بحث

عنوان البحث: سرطان الثدي الوراثي و التوصيف الجزيئي للجينات المرتبطة بها (BRCA1 ,BRCA2) وانتشارها بين النساء الفلسطينيات

الباحثون: نانسي جلة/ برنامج الماجستير في العلوم الطبية المخبرية/ جامعة بيرزيت و باشراف د. محمد فراج

حيثيات البحث : نرغب بمشارككم في هذا البحث من خلال الموافقة على أخذ كمية قليلة من الدم لعمل فحوصات للبحث عن الجينات التي لها علاقة بسرطان الثدي الوراثي .

### 1. الغرض والإجراءات المتبعة في البحث

• أهدف العام من المشروع هو تحديد مدى انتشار الجينات المرتبطة بسرطان الثدي الوراثي ( BRCA1, BRCA2 ) بين النساء الفلسطينيات.

### الاهداف المحددة هي:

1. تحديد التغييرات الجينية التي قد تساهم في ارتفاع نسبة سرطان الثدي الوراثي بين النساء الفلسطينيات.
2. امكانية الكشف المبكر عن طريق الفحص الجيني لتجنب الاصابة بسرطان الثدي الوراثي .
2. أية مخاطر يمكن توقعها : لا توجد مخاطر عدا عن الحد الأدنى من الراحة أثناء سحب الدم.
3. كيف سيتم الحفاظ على السرية: لن يتم استخدام اسم المشارك ، سيتم ترميز العينة.
4. عدد المشاركين : 100

إذا وافقت على المشاركة سوف تحصل على نسخة موقعة من هذه الوثيقة.

ان مشاركتك في هذا البحث سوف تساهم في زيادة المعرفة ونتائج هذه الدراسة سيكون لها تأثير ايجابي على الوضع الصحي في المجتمع الفلسطيني ولهذا نشكرك جدا على المشاركة.

توقيع المشارك: \_\_\_\_\_

رقم هاتف المشارك: \_\_\_\_\_

التاريخ: \_\_\_\_\_

**Annex 2: Primers used for amplification of 24 exons of BRCA1 and 27 exons of BRCA2:**

<b>Primer name</b>	<b>Sequence</b>
B2.2.F	TTCCAGGAGATGGGACTGAATTAG
B2.2.R	AAGCAACACTGTGACGTAAGTGGG
B2.3.F	GCCATCTTGTAACCTTTTGTGAACTC
B2.3.R.outer	CGTAATTCAGCAAATCTCAGTTGG
B2.3.R.seq	GTCTCCATTTTTTCGAGTGAGG
B2.4.F	GTAATCAGCAAACCTGAAAAACC
B2.4.R	GCCAATTCAACATCACAAGAAC
B2.5+6F	TACACGGTTTCCAGCAGC
B2.5+6R	GCAGAATGCTAGGTACAGATTTGTA
B2.7.F	TTATGACTTAGTAATTGAGAATTTG
B2.7.R	ATGTATTTGTATTTATTCCAATCTAC
B2.8.F	CCAACTCATTGTGGACAGTA
B2.8.R	CCAGGTTTAGAGACTTTCTCA
B2.9.F	CCACATCCTAGTGGTGCAAGATTTCC
B2.9.R	CTACTTGGCAAGCTGAGGCAGG
B2.10A.F	GCTTTACTAGAAGAACAGGAGAAGG
B2.10A.R	GGATCAGTATCATTGTTCCAC
B2.10B.F	GATACCTCTGAAGAAGATAGTT
B2.10B.R	GTATGAGATTCAAGATGCTG
B2.10C.F	GCATATTTCTTCATGTGACCA
B2.10C.R	GCCAGCTTCCATTATCAATTA
B2.10D.F	TTAAAAAAGAACTGAAGCCTC
B2.10D.R.outer	GTGACCTGATTCTAAACACTGG
B2.10D.R.seq	AAAAAAGACAGAGGTACCTGAATC
B2.11A.F	CCCAAAGTGCTGAGATTACAG
B2.11A.R	GAGTTAAAATAAGAGTGCTGGC
B2.11B.F	GGACAGTGTGAAAATGATCC
B2.11B.R	TGAGAAAAGTTCTTCAGAGTCTG
B2.11C.F	CCACCTGAAAAATACATGAGAG
B2.11C.R	GTGATTTGAAATTGGACCTAAG
B2.11D.F	ACCCAAGTGTCAATTAATAAAG
B2.11D.R	GTTTGAATTAATAATCCTGCTTG
B2.11E.F	CAAAGAACTGAGCAAGCC
B2.11E.R	TTGTGCCATGAGCAGAATAA
B2.11F.F	ACTTCTGAGGAATGCAGAGATG
B2.11F.R	TGGCAGCAGTATATTTGTTATC
B2.11G.F	GCAGAGGTACATCCAATAAG
B2.11G.R	GACTCTTTGGCGACACTAAT
B2.11H.F	AAGCATGTCATGGTAATACTTC

B2.11H.R	CTAATTCAAGGTCTTTACAGGC
B2.11I.F	GTGATGAAAAGATCAAAGAACC
B2.11I.R	TCAAGTAATGAAGTCTGACTCAC
B2.11J.F	AAACAGCAAAAAGTCCTGCAAC
B2.11J.R	GCTAGTCACAAGTTCCTCAACG
B2.11K.F	TGAGCCAGTATTGAAGAATGTTG
B2.11K.R	CTCCAATCCAGACATATTTTGG
B2.11L.F	CGAAAATTATGGCAGGTTGT
B2.11L.R	CTGTACTAAATCCAGAGAAAGCAGA
B2.11M.F	CGCAAGACAAGTGTTTTCTG
B2.11M.R	CCATTTCTGAGTTTACACAGTG
B2.11N.F	ATTTAGTACAGCAAGTGGAAAG
B2.11N.R	TTTTCTGAATCTTTGGAGTAAG
B2.11O.F	GGAAAAGAACAGGCTTCACCTA
B2.11O.R	ATCAAACCATACTCCCCCAA
B2.12.F	AAAACAGAACAATAATGTAATTG
B2.12.R	ATAAAGAGGTCCTTGATTAGGC
B2.13.F	ATTTATGCTGATTTCTGTTG
B2.13.R	TGTCTACTTTACAAAGCTGTC
B2.14A.F	ATATTGTTTTGGAATGGCAACC
B2.14A.R	TGCTTGATTGGAGTTGTTTTTG
B2.14B.F	GACACTTGATTACTACAGGC
B2.14B.R	GTAACAGCAGTCCTAGATTG
B2.15.F	GAGACAGGGTTTCTCCATTTTGG
B2.15.R	CATCCATTCCTGCACTAATGTGTTC
B2.16.F	CAGCAGACTGTGGAATGTATGGATC
B2.16.R	AAACCCCAGGACAAACAGCAC
B2.17.F	CACCATGCTCAGCAATGAAGT
B2.17.R	TGGGATGGCAACTGTCACTG
B2.18A.F	ATCCACTATTTGGGGATTGC
B2.18A.R	GAGGGGAGGATCTAACTGGG
B2.18B.F	TTTGTTTTCACTTTTATAGATATGATAC
B2.18B.R	TGTCTGAAGAATATGCTTTTTTATAC
B2.19.F	CATTAAGAACTTGTAGCAGTATAAAC
B2.19.R.outer	TGCGAAATATGTATAATCCAG
B2.19.R.seq	AAGATCACGCCATTGCACTC
B2.20.F.outer	TATCATCGCTTTTTCAGTGATGG
B2.20.R	TCCCTTGTTGCTATTCTTTGTC
B2.20.F.seq	GGCTGATCTCGAACTCCTGACC
B2.21.F	AACTTTTAGCAGTTATATAGTTTC
B2.21.R	AAATCCCAGAAATGTACAC
B2.22.F	TTAATAGTTTGAGGCACCTGAG

B2.22.R	AGTAGTGGATTTTGCTTCTCTG
B2.23.F	ATCAGAGAAGCAAAATCCAC
B2.23.R	CGACAAATCCTATTAGGTCC
B2.24.F	CATACAGTTAGCAGCGACAA
B2.24.R	GAGGTTCAAAGAGGCTTACTT
B2.25.F	GTCATTTTGGAAAACCTGAGCTTTC
B2.25.R	CATTCCCCATCTCCTGAGG
B2.26.F	CTCTCCCTATCAGCTAGATTCCCC
B2.26R	CTTACAGGAGCCACATAACAACCAC
B2.27A.F	ACCTATTAGGAGTTAGGGGAGG
B2.27A.R	GAACCAGACAAAAGAGCTT
B2.27B.F	CATTTTCAGCCACCAAGGAGTTG
B2.27B.R	TTTGCCCGATACACAAACGCTG
B1.1a.F	GCAATAAGCCGCAACTGGAA
B1.1a.R	TTCACAACGCCTTACGCCTC
B1.2.F	AAGGACGTTGTCATTAGTTCTT
B1.2.R	CATGTCTTTTCTTCCCTAGTATG
B1.3.F	AGGTAAGGATCGTATTCTCTGC
B1.3.R	GATTACAACCAACTTTTGATAAC
B1.5.F	AGGTTTTTGCTTATGCAGCA
B1.5.R	GCATTAGAGAAAGGCAGTAAGTTTC
B1.6.F	GGCTTAAATGAATGACAAAAAG
B1.6.R	AGATACAGAACTAAAATTAACCTAGAC
B1.7.F	CACAACAAAGAGCATACATAGGG
B1.7.R	GGAGGACTGCTTCTAGCCTG
B1.8.F	TGTTAGCTGACTGATGATGGT
B1.8.R	TTTGGCAAAACTATAAGATAAGG
B1.9.F	TTGTACCTGCCACAGTAGATGCTC
B1.9.R	CAAACCTGCACATACATCCCTGAAC
B1.10.F	TGGTCAGCTTTCTGTAATCG
B1.10.R	AGGTCCCAAATGGTCTTCAG
B1.11A.F	TAGCCAGTTGGTTGATTTCC
B1.11A.R	CTTCCAGCCCATCTGTTATG
B1.11B.F	CACAAATACTCATGCCAGCT
B1.11B.R	ACCTCATTTAGAACGTCCAA
B1.11C.F	GCAGCATTTCAGAAAGTTAATGAGTG
B1.11C.R	TCCGTTTGGTTAGTTCCCTG
B1.11D.F	CTGAATCAAATGCCAAAGTAGC
B1.11D.R	TGGGTTAGGATTTTCTCATTG
B1.11E.F	ATATCCACAATTCAAAGCACC
B1.11E.R	CAAAACCCTTTCTCCACTTAAC
B1.11G.F	GAAGGCTTTAAGTATCCATTGG

B1.11G.R	GACTTGATGGGAAAAAGTGGTG
B1.11H.F	GCCAGTTGATAATGCCAAATGTAG
B1.11H.R	GGATGCTTACAATTACTTCCAGGA
B1.11I.F	AACATTCAAGCAGAAGTAGG
B1.11I.R	GAAGCTCTTCATCCTCACTA
B1.11J.F	TTTGCTGAAAATGACATTAAGG
B1.11J.R	GTCACTCAGACCAACTCCCTGG
B1.11K.F	AGGCATCTCAGGAACATCAC
B1.11K.R	ATGAAAAGCACCTTAGGAGG
B1.12.F	GTCCTGCCAATGAGAAGAAA
B1.12.R	TGTCAGCAAACCTAAGAATGT
B1.13.F	TTAATTGCATGAATGTGGTTAG
B1.13.R	ATTAGTTGTGAGCAGGGACAAG
B1.14.F	CTAACCTGAATTATCACTATCA
B1.14.R	GTGTATAAATGCCTGTATGCA
B1.15.F	AGGCAACATGAATCCAGACTTC
B1.15.R	GAGCTATTTTTCTAAAGTGGGC
B1.16.F	AATTCTTAACAGAGACCAGAAC
B1.16.R	AAACTCTTTCCAGAATGTTGT
B1.17.F	AAATAGTTCCAGGACACG
B1.17.R.seq	TCCTAATCTCGTGATCTGCCCG
B1.18.F	GGCTCTTTAGCTTCTTAGGAC
B1.18.R	GAGACCCATTTTCCCAGCATC
B1.19.F	CTGTCATTCTTCCCTGTGCTC
B1.19.R	CATTGTTAAGGAAAGTGGTGC
B1.20.F	ATATGACGTGTCTGCTCCAC
B1.20.R	GGGAATCCAAATTACACAGC
B1.21.F	GCAGCAGAAATCATCAGGTG
B1.21.R	ATGCTCTTGAGAAGGGGGAC
B1.22.F	AGAGTACATGGCATATCAGTGG
B1.22.R	GTATGTGGGCAGAGAAGACTTC
B1.23.F.outer	TGAGCCTGGGAGCTGAAGAC
B1.23.R	GGAAACAGTTCATGTATTACTTTTAC
B1.23.F.seq	AAAAAATGATGAAGTGACAG
B1.24.F	ATGAATTGACACTAATCTCTGC
B1.24.R	GTAGCCAGGACAGTAGAAGGA