Biotechnology Master Program

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Biotechnology Master Program

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Two Novel BRCA1 and BRCA2 Mutations in Palestinian Women Affected with Breast Cancer

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By

Mohammad A. Salahat

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In Partial Fulfillment of the Requirements for the Degree of

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ABSTRACT

There has been a significant increase in breast cancer incidence in Palestine. Young age onset and cancer family history are suggestive of genetic predisposition. Molecular screening for BRCA1 and BRCA2 mutation is an established component of risk evaluation and management of familial breast cancer. Carriers of germ line mutations in these two genes are known to be at high risk of breast cancer. BRCA1 mutation carriers have an 18% risk (15% for BRCA2 mutation carriers) for developing breast cancer up to the age of 39 years, and the risk increases to 59% (34% for BRCA2 mutation carriers)) at ages 40–49 years. We have studied the family history of 365 Palestinian women affected with breast cancer. We have indentified those families with cancer history and utilized direct sequencing to screen for mutations in BRCA1 and BRCA2. A novel BRCA1 mutation, E203X in exon 10 was found in one patient of 365 probands with breast cancer. A novel BRCA2 mutation, E2229X in exon 11 was found in 3 unrelated patients. No carriers for E2229X mutation were detected among 362 probands with breast cancer. E2229X mutation did not appear among 1000 healthy Palestinian controls. We conclude that full BRCA1/2 screening should be offered to patients with characteristic family history.

إكتشاف طفرتين جديدتين في الجينين الأول والثاني لسرطان الثدي مع نساء فلسطينيات مصابات بهذا المرض

بواسطة

محمد علي صلاحات

ملخص

هنالك زيادة كبيرة في عدد حالات سرطان الثدي في فلسطين, وإن حدوث هذا المرض في سن مبكرة مع وجود تاريخ عائلي لهذا المرض يوحي بوجود إستعداد وراثي لهذا المرض. إن الفحص الوراثي للجينين, جين سرطان الثدي الاولBRCA1 و جين سرطان الثدي الثاني BRCA2 هو عنصر أساسي في تقيم المخاطر الوراثية, ومن ثم التعامل الصحيح مع سرطان الثدي الوراثي. من المعروف أن حاملي الطفرات الوراثية لهذين الجينين معرضين بنسبة عالية جدا للإصابة بسرطان الثدي الوراثي. لقد درسنا التاريخ العائلي للمرض ل 365 إمرأة فلسطينية مصابة بسرطان الثدي, وقمنا بإختيار عدد من تلك العائلات التي يوحي تاريخها العائلي بوجود طفرات وراثية في هذين الجينين , وأجرينا لها فحص تسلسل الأحماض النووية على المادة الوراثية المباشر للكشف عن وجود طفرات وراثية. لقد تم العثور على الطفرة E203X في الجين الأول في مريضة واحدة فقط من ال 365 مصابة بسرطان الثدي,و تم العثور على الطفرة E2229X في الجين الثاني في ثلاث مريضات من ال 365 مصابة بسرطان الثدي, ولم تظهر هذه الطفرة في 1000 إمرأة معافاة من سرطان الثدي كمراقبة لهذه الطفرة في النساء السليمات من المرض. نستنتج من ذلك أنه يجب إجراء الفحص الوراثي الكامل لهذين الجينين في مرضى سرطان الثدي الذين عندهم تاريخ عائلي مميز لهذا المرض.

DECLARATION

I declare that the Master Thesis entitled "Two Novel BRCA1 and BRCA2 mutations in Palestinian Women Affected with Breast Cancer" is an original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

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Date: July 7, 2011

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Dedication

This thesis is dedicated to my father, who taught me that there is nothing more valuable than learning, who supported me to reach this level. It is dedicated to my mother, who taught me that I should have real aim in my life, giving me all her love and support. It is dedicated to my wife Alaa,' for all of her love and support. With all love, this thesis is dedicated to my children Shahd, Lajayn, and Ali, source of happiness in my life. It is dedicated to all my family and friends who supported me. If I forget, I will never ever forget Martyrs of Palestine, for whom I dedicate this thesis.

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Abbreviations

Breast Cancer 1 Gene	BRCA1
Breast Cancer 2 Gene	BRCA2
Rad51 protein	RAD51
Polymerase Chain Reaction	PCR
Ataxia Telangiectasia Mutated	ATM
Checkpoint Kinase 1	CHK1
Tumor protein 53	p53
Retinoblastoma protein	RB
FANCD2 protein	FANCD2
BRCA1-associated RING domain 1	BARD1
BLM protein that is affected in Bloom syndrome	BLM
MSH2 Protein	MSH2
MSH6 Protein	MSH6
MRE11 Protein	MRE11
RAD50 Protein	RAD50
Nijmegen breakage syndrome 1	NBS1
X-inactive specific transcript	*XIST*
BACH1 protein	BACH1
SW1 Protein	SW1
SNF Protein	SNF
Histone Deacetylases	HDAC
Checkpoint Kinase 2	CHK2

Polo-like Kinase 1	PLK1
GADD45 Protein	GADD45
BAP1 Protein	BAP1
E2F1 Protein	E2F1
Mitomycin	MYC
ZBRK1 Protein	ZBRK1
Nuclear Localization Signals	NLSs
BRCA1-Associated Surveillance Complex	BASC
MDC1 Protein	MDC1
Cycle-Dependent Kinase 2	CDK2
Clusters of serine and threonine sequences	SQ sequences
SQ-Cluster Domains	SCDs
BRCA1 Carboxyl Terminus	BRCT
Deoxyribonucleic Acid	DNA
Ribonucleic Acid	RNA
BACH1 Protein	BACH1
HDAC1 Protein	HDAC1
Carboxy-terminal-binding-Protein	CtIP
DSS1 Protein	DSS1

List of Figures

Figure	Description	Page
1	The BRCA1 network	8
2	BRCA1 and BRCA2 functional domains	10
3	A pedigree showing a famiy history of breast cancer	20
4	Identification of the novel E 203 X mutation	31
5	Family pedigree of the patient, has E 203 X mutation	32
6	Identification of the novel E 2229 X mutation	33
7	Family pedigree of the patient, has E 2229 X mutation	34
8	Identification of the frame-shift 2482delGACT mutation	35
9	Family pedigree of the patient, has frame-shift 2482delGACT mutation was	36
10	Digestion of E2229X mutation	37
11	Family pedigree of the Breast Cancer patient, carrier for E 2229 X mutation	38
12	Family pedigree of the Breast Cancer patient, carrier for E 2229 X mutation	39
13	Six flankig markers around E 2229 X- BRCA2 mutation	40
14	Control result for 2482delGACT-BRCA2 mutation	41
15	Result for patient with heterozygous 2482delGACT-BRCA2 mutation	42
16	Family pedigree of the patient, carrier for 2482delGACT mutation	43
17	Family pedigree of the patient, carrier for 2482delGACT mutation	44

List of Tables

Table	Description	Page
1	Clinical characteristics of the population cohort	17

Table of Contents

ABSTRACT II

Dedication VI

Acknowledgment VII

Abbreviations VIII

List of Figures X

List of Tables XI

CHAPTER 1 1

BACKGROUND 1

1.1 Breast Cancer 1

1.2 Breast cancer statistics 2

1.3 Risk Factors of breast cancer 3

1.4 Hereditary Breast Cancer 5

1.4.1 BRCA1 and BRCA2 5

1.4.2 Functions of BRCA1 and BRCA2 6

1.4.3 BRCA1 and BRCA2 mutations 12

1.4.4 Founder effect 13

CHAPTER 2 14

Literature Review 14

Objectives 16

CHAPTER 3 17

Methodology 17

3.1 Data and Pedigree Construction 17

3.2 DNA Extraction....18
3.3 Sequencing....19
3.3.1 PCR protocol. 21
3.3.2 PCR product Purification 21
3.3.3 PCR Sequencing Protocol 22
3.3.4 PCR Sequencing Product Purification 23
3.4 RFLP testing 24
3.4.1 PCR protocol 24
3.4.1 PCR Cocktail 25
3.4.2 Restriction Digest Protocol....25
3.5 Genotyping Analysis 26
3.5.1 PCR cocktail....26
3.5.2 Genotyping cocktail 27
3.6 Haplotype Analysis 28
5.6.1 PCR Protocol....28
3.6.2 Genotyping Protocol 30
CHAPTER 4.... 31
Results....31
4.1 Sequencing Results....31
4.1.1 E203X mutation....31
4.1.2 E2229X mutation....33
4.1.3 2482delGACT mutation....35
4.2 Screening Result....37
4.3 Haplotyping Results 39
4.4 Genotyping Results 41

CHAPTER 5..... 45
Discussion 45
5.1 Study significance 46
Conclusion.....47
Appendix A 48
Appendix B 52
References 55

CHAPTER 1

BACKGROUND

1.1 Breast Cancer

Breast cancer is one of the most common malignancies affecting women in the developed countries. In Palestine, there are many cases of breast cancer that have been diagnosed every year. According to the Palestinian Health Information Center, and Ministry of Health – Nablus, Breast Cancer is the first cancer type in number of cases; there are 189 cases, during 2009. Breast Cancer is the third cancer type in number of deaths; there are 67 deaths during the same year. This type of cancer begins when normal cells in the breast start to grow and divide uncontrollably, producing a mass called tumor. A tumor can be benign (noncancerous, localized) or malignant (cancerous, metastasized). The breast is a highly modified glandular organ, composed of fatty tissue, surrounding a network of lobes which are composed of minute tube-like structures called lobules that hold milk glands. Small ducts connect the milk glands, lobules, and lobes, transporting the milk from the lobes to the nipple, placed in the middle of the areola which surrounds the nipple [1].

Approximately 75% of all breast cancers begin in the cells coating the milk ducts which are called ductal carcinomas in situ (DCIS). Fifteen percent of breast cancers begin in the lobules, and are called lobular carcinoma in situ (LCIS). Spreading of ductal carcinoma outside the duct is called invasive ductal carcinoma, and spreading of lobular carcinoma

outside the duct is called invasive lobular carcinoma. Otherwise it is called in situ ductal or lobular carcinoma. Presently, oncologists advise that DCIS, the major type of breast cancer, is to be surgically removed in order to help preventing the breast cancer from becoming an invasive breast cancer. In addition to surgery, hormonal therapy and radiation therapy can be used. There are other less common types of breast cancer such as mucinous, medullary, tubular, papillary, metaplastic breast cancer and inflammatory breast cancer which is considered the faster-growing type of breast cancer, responsible for 1%- 5% of all breast cancer cases [2].

1.2 Breast cancer statistics

The American Cancer Society estimates that there will be about 207,090 new cases of invasive breast cancer diagnosed in 2010. 39,840 expected breast cancer deaths will occur this year among women in the United States. The percentage of people who stay alive at for least five years after diagnosis is 98% if the cancer is limited to the breast, 84% if the cancer is extended to the local lymph nodes, and 23% if the cancer is spread systematically.

In the United states, since 1990, there is a steady decrease in the number of women who have died of breast cancer each year. There has been a decrease of about 3% per year in women younger than 50 years old; and 2% per year in women older than 50 years old. Presently, it has been estimated that more than two and a half million women living in the United States who have been diagnosed and treated for breast cancer [2].

1.3 Risk Factors of breast cancer

Risk factors increase the chance of developing cancer. Many women develop breast cancer while they don't have any obvious risk factor. Some women don't develop breast cancer even when they have known risk factors. There are many risk factors of Breast Cancer. The following are some important risk factors which may increase a woman's risk for developing breast cancer:

Age: The risk of developing breast cancer increases as women get older, the majority of breast cancer cases are in women over 50. Lifetime risk of a woman reaches to age 90 is about 14.3%, or one in seven [3].

Gender: According to the American Cancer Society being a woman is the major risk factor for developing breast cancer, **due to** continuous exposure to the growth-promoting effects of the female hormones estrogen and progesterone. Breast cancer is 100 times more frequent among women than men.

Race and ethnicity: According to the American Cancer Society, the prevalence of cancer in the United States is highest in African-Americans followed by Caucasians, Hispanics, Asian-Americans and American Natives, and the percentage of those die from breast cancer is highest in African-Americans followed by Caucasians, American Natives, Hispanics and Asian-Americans [4].

Estrogen and progesterone exposure: Women who starts menstruating at an early age, before age 12, or get a delayed menopause, after age 55, have a higher risk of breast cancer, due to long time exposure to estrogen and progesterone [5].

Personal history of breast cancer: According to the American Cancer Society, a woman who has had breast cancer in one breast has a 1% to 2% possibility per year of developing a second breast cancer in her other breast [2].

Family history of breast cancer: Breast cancer risk is higher among women who have a first-degree relative (mother, sister, and daughter) diagnosed with breast cancer, this elevates the risk about 2-folds. Having more than one first-degree relative with breast cancer elevates the risk about 3-folds, particularly if the cancer occurred in early age. Having a second-degree relative (grandmother, granddaughter, and aunt) also increases the risk. The father's (paternal) side of the family should also be considered equally to the mother's (maternal) side when evaluating a family history. Less than 15% of women diagnosed with breast cancer have a family history of Breast Cancer [2].

Genetic risk factors: Hereditary Breast Cancer may be responsible for 5-10% of breast cancer cases [6]. The following section will clarify the meaning of Hereditary Breast Cancer, and elaborate on the most important genes involved.

1.4 Hereditary Breast Cancer

Approximately 5% to 10% of breast cancer cases are thought to be hereditary, resulting directly from mutations inherited from parents. Germ line mutations in BRCA1 and BRCA2 account for the vast majority of families with hereditary breast cancer [7].

Persons with hereditary breast cancer inherit one defective allele in BRCA1 or BRCA2 from their father or mother, but the second copy of allele is functional. If this functional allele becomes defective, cancer can develop through the accumulation of additional mutations. This hypothesis is called the “two-hit hypothesis” [8].

Generally, about 1 in 300 to 1 in 800 individuals carry a mutation in BRCA1 or BRCA2 [9]. In certain populations such as Ashkenazi Jews, this percentage decreases to be about 1 in 40, due to founder effect of these mutations. It has been estimated that the lifetime risk of breast cancer due toBRCA1 or BRCA2 mutation is 65–74% [10], [11].

Inherited mutations in other genes also increase the risk for developing breast cancer including the cell cycle regulator CHEK2 gene involved in increasing the risk for developing breast cancer 2-fold [12], [13]. Inherited mutations of TP53 in families with Li-Fraumeni syndrome and of PTEN in families with Cowden syndrome highly increase the risk for developing breast cancer, particularly early onset breast cancer [14], [15].

1.4.1 BRCA1 and BRCA2

About 16 years have passed since Mark Skolnick and his colleagues at Myriad Genetics in Salt Lake City (Utah, USA) published in 1994 that they had cloned BRCA1 [16]. First

evidence for the existence of the gene was provided by the King lab at UC Berkeley in 1990 [17]. The human BRCA1 gene is situated on the long (q) arm of chromosome 17 at band 21, from base pair 38,429,551 to base pair 38,551,283.

As a conclusion of massive research on male breast cancer families, which were found not to carry BRCA1 mutations, and by using linkage analysis, BRCA2 gene was mapped in 1994 and cloned in 1995 at the Institute of Cancer Research, UK. BRCA2 gene is on the long (q) arm of chromosome 13 at position 12.3 (13q12.3), from base pair 31,787,616 to base pair 31,871,804 [18].

1.4.2 Functions of BRCA1 and BRCA2

BRCA2 protein has more limited function than BRCA1 protein. BRCA1 and BRCA2 proteins are involved in DNA repair, particularly homologous recombination, cell cycle checkpoint control, chromatin remodeling and protein ubiquitylation [19].

1.4.2.1 DNA Repair

BRCA1 and BRCA2 proteins are involved in DNA repair by homologous recombination. Once DNA is damaged, both BRCA1 and RAD51 proteins localize to the damaged area. BRCA2 interacts directly with RAD51, through its carboxyl terminus and its BRC repeats, forming a complex [20], that is believed to interact with FANCD2 which binds to BRCA1, that is phosphorelated in this process, see figure 1.

BRCA1 directly binds to DNA, with higher affinity for branched DNA structures. This ability to bind to DNA contributes to its ability to inhibit the nuclease activity of the MRN complex as well as the nuclease activity of Mre11 [21]. This may explain a role

for BRCA1 to promote higher fidelity DNA repair by non-homologous end joining (NHEJ) [22]. BRCA1 also co-localizes with γ-H2AX (histoneH2AX phosphorylated on serine-139) in DNA double-strand break repair foci, indicating it may play a role in recruiting repair factors [23].

Genomic DNA with malfunctioning BRCA1 or BRCA2 is susceptible to factors that create breaks in double-stranded DNA such as mitomycin and cisplatin [24]. In these cells double strand breaks are repaired by an error-prone mechanism, such as NHEJ, leading to chromosomal rearrangements [25], [26]. These errors in chromosomal rearrangements cause chromosomal instability, the main feature of carcinogenesis.

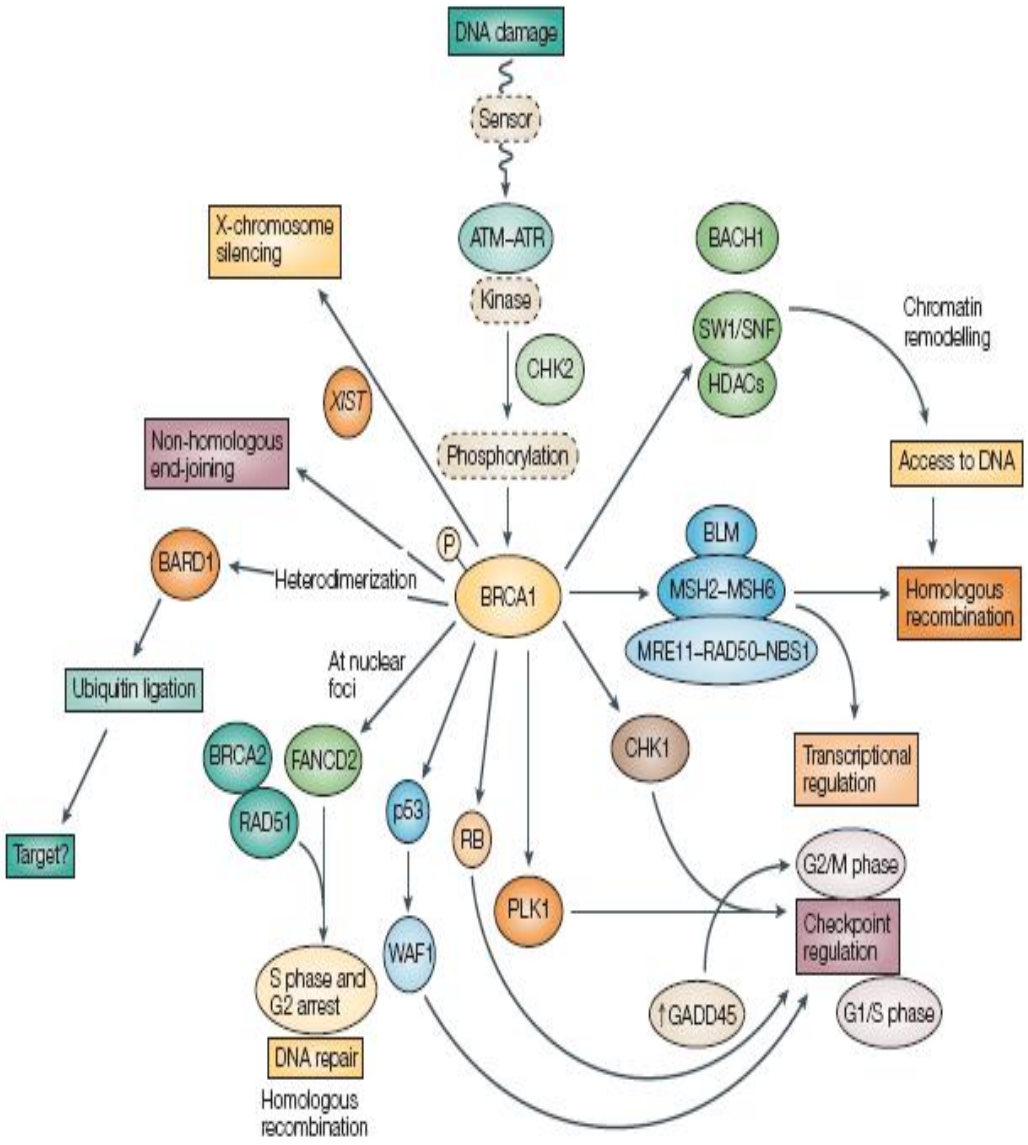


Figure 1: The BRCA1 network, this figure clarifies how BRCA1 is an important component of pathways that regulate DNA repair, cell-cycle progression, ubiquitylation and transcriptional regulation, and the role of BRCA1 and BRCA2 in DNA repair [27].

Cells that have defective BRCA1 and BRCA2 are sensitive to ionizing radiation and demonstrate error-prone repair. It has been proved that BRCA2 and RAD51 proteins increase in cells when they go into S phase, signifying that they function during or after DNA replication. Consequently, BRCA1 and BRCA2 have a main role in a common pathway that is accountable for the integrity of the genome and the protection of chromosomal stability [28].

BRCA1 is not only involved in the repair of double strand breaks, moreover it might be involved in nucleotide-excision repair. Nucleotide excision repair enzymes recognize distortion sites in DNA double helix. Recognition of these distortions leads to the removal of a short single-stranded DNA segment that includes the damage, creating a single-strand gap in the DNA, which is subsequently filled in by DNA polymerase, which uses the undamaged strand as a template [29].

This type of repair includes two different methods; one is transcription-coupled repair, in which the transcribed strand of DNA is primarily repaired. In cells with defective BRCA1 the transcription-coupled repair might be restricted to the blockage of the RNA polymerase II transcription machinery at the place of repair of 8-oxoguanine residues [30]. The second is global genome repair, by which damage in both transcribed and untranscribed DNA strands is repaired. By this mechanism, several 'damage-sensing' proteins, including the DNA-damage binding (DDB) and XPC-Rad23B complexes that constantly scan the genome and recognize helix distortions, and subsequently repair proteins, are then recruited to the damaged DNA to verify presence of DNA damage [29] .

1.4.2.2 Checkpoint Control

Cell cycle checkpoint control is another important function of BRCA1 (figure 1). BRCA1 is an important component of the BRCA1-associated genome-surveillance complex (BASC) [31]. BRCA1 has the BRCT motif at the carboxyl terminus (figure 2) which is a common feature of proteins that are implicated in DNA repair and/or cell-cycle checkpoints. Interestingly, unlike BRCA1, the checkpoint function is preserved in BRCA2-deficient primary cells.

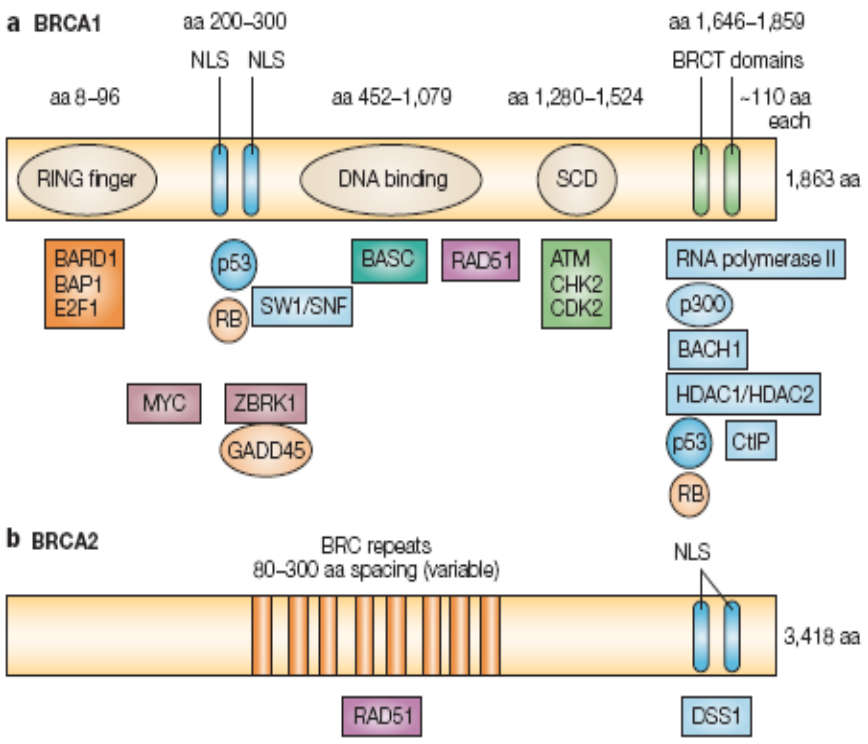


Figure 2: BRCA1 and BRCA2 functional domains, and selected binding partners [27].

Mice defective for BRCA1 and BRCA2, died during early phases of embryogenesis but loss of p53 or WAF1 (also known as p21) delays this embryonic lethality by a few days [32]. The majority of cells that have defective BRCA1 and BRCA2 undergo apoptosis because of checkpoint controls, but if cells with defective checkpoint proteins such as p53 or WAF1 have defective BRCA1 or BRCA2, cells survive in the presence of genomic instability**.** This results in the typically abnormal karyotypes that are seen in breast cancers associated with mutations in BRCA1 and BRCA2 [33].

1.4.2.3 Ubiquitylation

Ubiquitination is an enzymatic reaction that tags protein with ubiquitin and thus distant a protein in a post-translational modification process for degradation. BRCA1 shares a ring finger with ubiquitinating proteins on its n-terminal. It has been revealed that the N-terminus of the BRCA1 protein functions as an E3 ubiquitin ligase, having the ability to catalyze monoubiquitin and polyubiquitin targetting. BRCA1 ubiquitination activity is greatly increased when BRCA1 is in a complex with its N-terminal binding partner BAR1.

BRCA1-mediated ubiquitination occurs in response to replication stress and DNA damage, indicating that there is some relationship between ubiquitination activity of BRCA1 and DNA-damage response [34].

1.4.2.4 Chromatin Remodeling

Chromatin remodeling process includes the efficient shifting of nucleosome cores along the extent of the DNA molecule, a process known as “nucleosome sliding”. Nucleosomes reduce the binding of a variety of DNA regulatory proteins including transcription factors, DNA repair components, and proteins of recombination machinery. This process takes place around double-strand DNA breaks and is believed to assist DNA repair. BRCA1 protein is involved in a number of chromatin remodeling complexes such as BRCA1-associated genome-surveillance complex (BASC), SW1 and SNF (figure 2) [35].BRCA1 is also implicated in activation process of genes that are involved in response to DNA damage, such as KU70 and GADD45 [36]. Interestingly, a BRCA2-interacting protein, EMSY77, also has DNA repair functions, and mutations that affect this protein might underlie sporadic breast cancer [37].

1.4.3 BRCA1 and BRCA2 mutations

The majority of BRCA1 mutations cause protein truncations; either small insertions or deletions or nonsense mutations leading to forming stop codons [38]. Large deletions in BRCA1 are uncommon, comprising 5–10% of all germ line mutations, and these mutations are even less common in BRCA2 [39]. In rare cases, complex rearrangements that involve BRCA1 which involve repetitive elements, such as *Alu* sequences were reported [40]. More than 300 missense mutations in BRCA1 have been reported in database [41]. These mutations cause alteration in a single amino acid, leaving the protein intact. The effect of these mutations on protein function is not well understood.

They are considered as a challenging investigation for scientists to evaluate the risk of developing cancer in a woman who is found to carry missense mutation. There have been in vitro attempts to study the effect of these variants on protein function; however its clinical impact remains elusive.

1.4.4 Founder effect

Some BRCA1 and BRCA2 mutations are profiled in specific geographic and/or ethnic populations. These mutations are called founder mutations, due to their founder inheritance. Simply founder mutations are the mutations spread from a single ancestor. Since the identification of BRCA1 and BRCA2 genes, many researchers have been studying founder mutations in different populations. First and most common fonder mutations of BRCA1 and BRCA2 were identified by Simard and colleagues in 1994 [42]. They had identified BRCA1 185delAG and 5382insC as recurrent mutations from Quebec, Canada with strong Ashkenazi ancestry. Offit and Neuhausen identified yet 6174delT mutation as founder mutation of BRCA2 with Ashkenazi Ancestry [there is no Jewish ancestry, Sephardi and Mizrachi Jews are different]. One of the three founder mutations is present in 2% of Ashkenazi Jews [43]. Consequently, many researchers consider testing for only these three founder mutations in Ashkenazi Jewish women with breast cancer.

Founder mutations have also been identified in Icelandic, Dutch, Norwegian, French Canadian [44], Austrian [45], Belgian [46], Finnish [47], British [48], and Swedish [49] populations.

CHAPTER 2

Literature Review

After the localization [17], [18]and isolation [16], [50] of the breast cancer susceptibility genes BRCA1 and BRCA2, researchers started to study the mutation prevalence of these two genes in Breast Cancer patients. They started to identify inherited mutations of BRCA1 and BRCA2, then estimating the contributions of these mutations to breast cancer risk in different populations.

Simard and colleagues (1994) identified two BRCA1 mutations (185delAG and 5382insC), in Ashkenazi Jews [42]. Two years later, Offit and Neuhausen (1996) identified 6174delT also in Ashkenazi Jews in BRCA2. Mutations in the BRCA1 and BRCA2 genes are found in most families with multiple cases of breast cancer.

Ford and colleagues studied the contribution of BRCA1 and BRCA2 mutations to inherited breast cancer, in 237 multiply affected families, each family with at least four cases of breast cancer, without regard to the occurrence of ovarian or other cancers [7]. They found that breast cancer was linked to BRCA1 in an estimated 52% of families, to BRCA2 in 32% of families, and to neither genes in 16%, suggesting other predisposition genes. The majority (81%) of the breast-ovarian cancer families were due to BRCA1, with most others (14%) due to BRCA2. Conversely, the majority of families with male and female breast cancer were due to BRCA2 (76%). The largest proportion (67%) of

families due to other genes was found in families with four or five cases of female breast cancer only.

In order to study the prevalence of BRCA1 and BRCA2 genes mutations in patients with early-onset breast cancer, Julian and colleagues (1999) carried out a population-based study of young patients with breast cancer from Britain. They detected mutations in 15 (5.9%) of 254 women diagnosed with breast cancer before age 36 years (nine [3.5%] in BRCA1 and six [2.4%] in BRCA2) and in 15 (4.1%) of 363 women diagnosed from ages 36 through 45 years (seven [1.9%] in BRCA1 and eight [2.2%] in BRCA2). Eleven percent (six of 55) of patients with a first degree relative who developed ovarian cancer or breast cancer by age 60 years were mutation carriers, compared with 45% (five of 11) of patients with two or more affected first or second-degree relatives, revealing that BRCA1 and BRCA2 genes make approximately equal contributions to early-onset breast cancer in Britain [51].

King and colleagues studied breast cancer risks in Ashkenazi Jewish women with inherited mutations in BRCA1 and BRCA2. They selected 1008 index cases regardless of family history. They found that the lifetime risk of breast cancer among female mutation carriers was 82%, similar to risks in families with many cases. Risk appears to increase with time, breast cancer risk by age 50 among mutation carriers born before 1940 was 24%, but among those born after 1940 it was 67% [11].

Finally, Kadouri and colleagues (2007) studied 31 women of Palestinian Arab origin affected with breast (n = 28), ovarian (n = 3) cancer. They found a novel BRCA1 mutation, E1373X in exon 12, in a patient affected with ovarian cancer. Four of her family members, three breast cancer patients and a healthy individual were consequently also found to carry this mutation [52].

Objectives

1. Identification of BRCA1 and BRCA2 mutations in Palestinian breast cancer patients with family history.
2. Detection of the prevalence of these mutations in a cohort of Palestinian breast cancer patients.

CHAPTER 3

Methodology

3.1 Data and Pedigree Construction

Three hundred and fifty blood samples of Palestinian women affected with breast cancer were collected from Augusta Victoria hospital, during 2007–2010. All participants signed an informed consent form approved by Bethlehem University's Institutional Review Board (IRB) and the institutional ethics committee at Augusta Victoria hospital. Each proband was inter-viewed and family pedigree was constructed utilizing (progeny software). Clinical characteristics of the study population are presented in table 1.

Table 1: Clinical characteristics of study population

Index Cases				Mean Age at Diagnoses	Family history of breast cancer N(%)	Family history of ovarian cancer N(%)	Family history of other cancers N(%)
Number(N) of families	Breast Cancer N(%)	Ovarian Cancer N(%)	Breast + Ovarian N(%)				
365	355 (97.3%)	10 (2.7%)	1 (0.3%)	47.4 yrs	96 (26.3 %)	6 (1.6%)	184 (50.4%)

Of 365 samples, family history of Breast Cancer was recorded in 96 (26.3 %), family history of ovarian cancer was recorded in 6 (1.6%), and family history of other cancers (pancreas, prostate, leukemia, lymphoma, lung, and others) was recorded in 184 (50.4%). Mean of age at diagnosis is 47.4 yrs.

3.2 DNA Extraction

DNA was extracted according to the salt precipitation protocol as follow:

1. 5 ml of intravenous blood was collected in EDTA tubes, and lysed in 45 ml RBCs lyses buffer on ice for 30 min.
2. Centrifuged at 2000rpm for 10 min at 4ºC.
3. Supernatant was removed. The pellet was washed carefully with 10ml of RBCs lyses buffer RBC buffer.
4. The pellet was broken and mixed with 50µl of 5mg/ml proteinase K. +100ul 20% SDS+3ml DNA lyses buffer and incubated with shaking for 2 days at 37ºC water.
5. 1ml of 6 M NaCl was added and mixed.
6. Centrifuged at 3000 rpm for 20 min at 25ºC for two times.
7. 100% of ice cold ethanol was added. The volume of ethanol should be twice the volume of supernatant in the tube.
8. The tube was very slowly inverted to collect the DNA
9. DNA was removed with a clean Pasteur pipette.
10. DNA was washed with 70% cold ethanol, and then dried on air for 3-5 min.
11. DNA was dissolved in DD Water, with 0.02% Sodium Azide.
12. The concentration of the DNA was checked and diluted to working concentration of 100ng/µl.

13 Genomic DNA was kept at -20ºC.

3.3 Sequencing

DNA of 16 probands was chosen for full sequencing of both BRCA1 and BRCA2 genes\ based on the following criteria

1. Multiple cases of early-onset of breast cancer (< 50 years old)
2. Ovarian cancer (with family history of breast or ovarian cancer)
3. Breast and ovarian primary cancers in the same woman
4. Male breast cancer

The pedigree of one of these 16 samples is presented in figure 3.

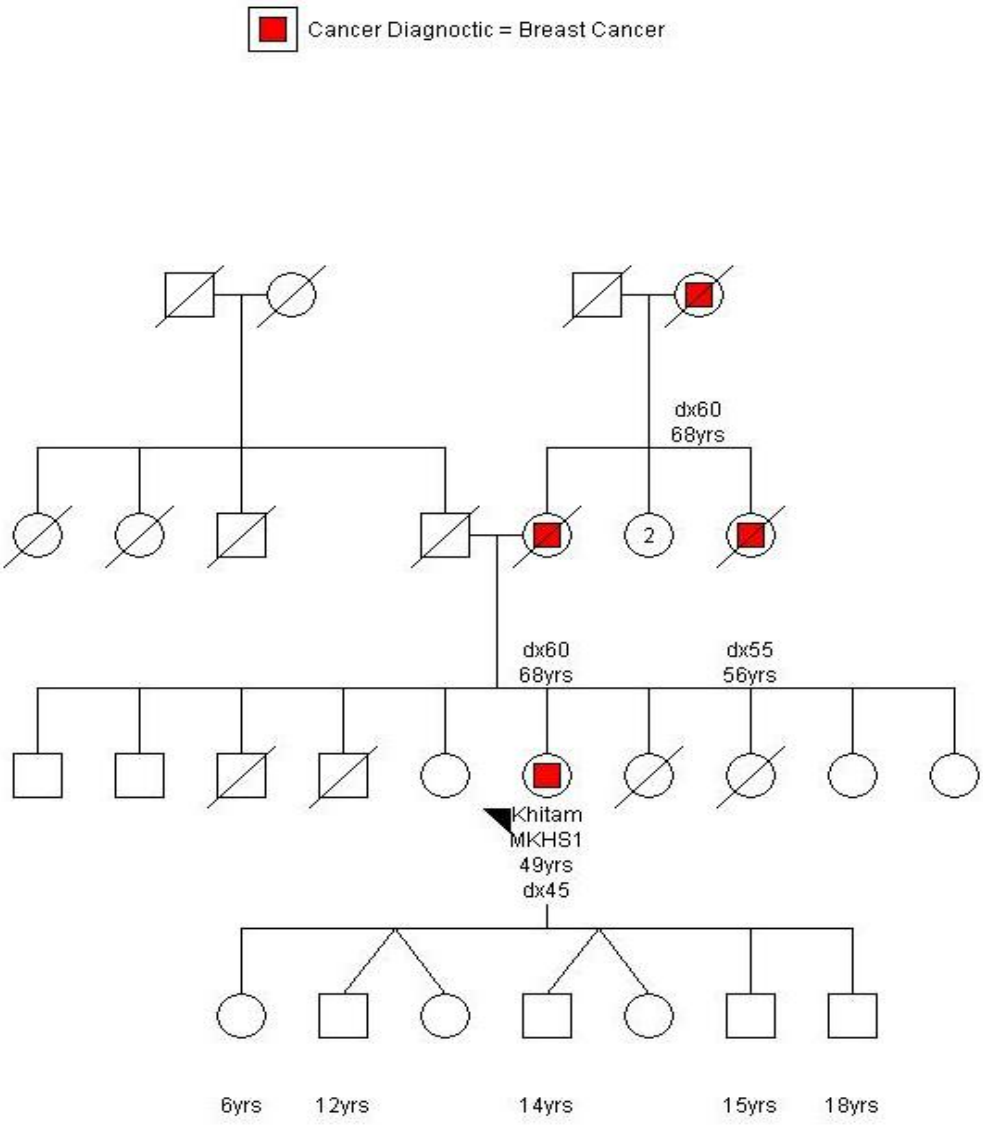


Figure 3: A Pedigree showing a family history of breast cancer.

Full sequencing of BRCA1 and BRCA2 genes was performed according to Applied Biosystem Sequencing Protocol.

3.3.1 PCR protocol.

	1X
Ready mix:	12.5 µl (Thermo Fisher Scientific/ Cat # AB-0575/DC/LD/B)
Forward Primer:	0.5µl from (10 pmol / µl).
Reverse Primer:	0.5µl from (10 pmol / µl).
Template:	0.5 µl
D.D H_2O:	up to 25 µl

Primers used for amplification of 24 exons of BRCA1 and 27 exons of BRCA2 are presented in Appendix A. PCR programs for all the primers are presented in Appendix B.

3.3.2 PCR product Purification

PCR product was purified, using separate Exonuclease1 (Biolabs, New England, United States/cat# M0293L) and Antarctic phosphatase PCR clean-up method (Biolabs cat# M0293L) as follow:

a) Exo1- Antarctic Phosphatase Master Mix was prepared as follows: 0.25 µl Exonuclease Conc. : 20.000U/ml + 0.25 µl Antarctic Phosphatase Conc. : 5.000U\ml +1.8 µl d.d H2O

b) Aliquot of 2.0 µl of the Master Mix was used for each 5.0 µl PCR product in a PCR plate.

c) PCR plate was placed into thermocycler (Gene Amp, PCR System 9700, Applied Biosystem), according to the following programme:

37 ºC for 30 minutes (enzyme incubation).

80 ºC for 20 minutes (enzyme inactivation).

4 ºC for a minimum of 5 minute.

Then cleaned PCR products were sequenced as follow:

3.3.3 PCR Sequencing Protocol

1X

Big Dye: 1 µl Big Dye Terminator V 1.1(Applied Biosystem P/N: 4336791

5XBuffer: 4 µl (Big Dye Buffer 0612)

Primer (F or R): 1µl from (10 pmol /µl).

Template: According to the template size and concentration.

H_2O: up to 20 µl

3.3.4 PCR Sequencing Product Purification

The sequencing PCR product was cleaned using EDTA/Ethanol Precipitation method (Applied Biosystem) as follows:

a) 5.0µl 125mM EDTA was added to each well contains 20.0µl of sequencing PCR product
b) 100µl of 100% Ethanol was added to each well.
c) Incubation at room temp, in dark for 1hr.
d) Centrifuged at 4 ºC, 3800rpm, for 30min.
e) Supernatant was discarded.
f) 60 µl of 70% Ethanol was added to each well.
g) Centrifuged at 4 ºC, 3800rpm, for 30min, Strips contents were discarded, inverted onto paper towel and centrifuged at 4 ºC, 500rpm, for 2min.
h) Strips were dried at 95 ºC on hotplate for 5min.

Denaturation: 16 µl of deionized Formamide (Applied Biosystem P/N#:4311320,) was added to the dried product, mixed well, spinnend down, denatured at 95C for 2min put on ice for 5 min. and then sequenced on the automated ABI 3130 Genetic Analyzer (S/N:20355-023, Applied Biosystem/HITACHI).

Then Sequences were screened for mutations, using **SeqScape** Analysis Software v25. (P/N: 4363009 Rev.A, Applied Biosystem).

3.4 RFLP testing

RFLP (Restriction fragment length polymorphism) analysis was used as a screening protocol for the novel BRCA2 G to T (E2229X) mutation - in 365 samples. **BfaI** enzyme has been used for enzymatic digestion as follow:

3.4.1 PCR protocol

The forward and reverse primers were:

E2229X-F: 5'GTCAGAAAACTACTTTGAAACAGAAGCACTA 3' , and

E2229X-R: 5' AACCATTTCCTCATTTTCGGGAC 3', and yielding a product size 136 bp. The PCR programme was**:**

Step1:	**95** ºC for 4 min.
Step2 (3 cycles):	**94** ºC for 30 sec, **58** ºC for 30 sec, **72** ºC for 30 sec
Step3 (3 cycles):	**94** ºC for 30 sec, **56** ºC for 30 sec, **72** ºC for 30 sec
Step4 (3 cycles):	**94** ºC for 30 sec, **54** ºC for 30 sec, **72** ºC for 30 sec
Step5 (3 cycles):	**94** ºC for 30 sec, **52** ºC for 30 sec, **72** ºC for 30 sec
Step6 (35 cycles):	**94** ºC for 30 sec, **50** ºC for 30 sec, **72** ºC for 30 sec
Step7:	**72** ºC for 5 min, **4** ºC for 10 min.

3.4.1 PCR Cocktail

	1X
Ready mix:	12.5 µl 12.5 µl (Termo Fisher Scientific Cat # AB-0575/DC/LD/B)
Forward Primer:	0.5µl from (10 pmol / µl).
Reverse Primer:	0.5µl from (10 pmol / µl).
Template:	0.5 µl
D.D H_2O:	up to 25 µl

3.4.2 Restriction Digest Protocol

	1X
Buffer 4 (10X, Biolabs, New England, United States #B70045):	2 µl
BfaI enzyme (Biolabs New England United States #RO568L, 5000U/ml)**:**	1 µl
PCR product:	3 µl
D.D H_2O:	up to 20 µl

Incubation: at 37 ºC, over night

Running; on 4% agarose gel

3.5 Genotyping Analysis

I used this procedure as a screening test for 2482delGACT mutation in all 365 samples, using fluorescent labeled forward primer according the following protocol:

1. **PCR for all samples as follow:**

3.5.1 PCR cocktail

	1X
10X buffer: Batch #:290112:	1.5µl
(**25mM) Mgcl2**: B. #: 290112:	1.0µl
dNTBs (Lot # B8901-1, 2.5mM each\800ML):	1.0µl
Taq DNA polymerase (5U\ML, Lot No. : TP\024\2001\17 Cat #: AB-1192\A)	0.05µl
F Primer:	0.25µl
R Primer:	0.25µl
DNA (100ng/ µl)**:**	0.3µl
D.D H2O:	8.4µl

PCR program: T.D 50-30

Primers: **BRCA2-MKG-*F** - ACAGAACATCCTTGGAAGTAG -3' FAM or Hex

BRCA2-MKG-*R - GTTTCTTAGTTATTTATTACCCCAGAAGC -3'

2. **PCR product was diluted with D.D water, 1:40.**

3. **Then genotyped as follow:**

3.5.2 Genotyping cocktail

	1X
Hi-Di-Formamide (P/N: 4311320, L/N: 1002928)**:**	10.7µl
Rox (P/N: 402985 L/N: 1005364)**:**	0.3µl
PCR product (diluted)**:**	1.0µl

4. **Running on ABI 3130 Genetic Analyzer (S/N: 20355-023).**
5. **Then results were analyzed using Genotyper software (P/N: 4326570 Rev.A, Applied Biosystem).**

3.6 Haplotype Analysis

In order to establish that a shared mutation has a common founder effect, six fluorescent microsatellite markers flanking the mutation were genotyped utilizing ABI 3130 Gene Mapper software, according to the following ABI-Genotyping protocol:

1. **PCR was done for three samples, carrying E2229X mutation as follow:**

5.6.1 PCR Protocol

	1X
Taq DNA polymerase (5U\ML, Cat #: AB-1192\A, Fisher)	0.5µl
10X buffer:	1.5µl
(**25mM) Mgcl2**:	1.0µl
dNTBs (2.5mM each\800ML):	2.5µl
F Primer:	1.0µl
R Primer:	1.0µl
DNA (100ng/ µl)**:**	1.0µl
D.D H2O:	14.5µl

Primers of the six Markers:

Primer	Size(bp)	Tm(°C)-cycles
BRCA2 TG1-F in FAM: GGTTGCATTCTTGATTGGCTA BRCA2 TG1-R: GGCTCACATTGAAGCACAAA	180	58-35
BRCA2 TG2-F in FAM: TTTGTGTTCCAGGTGAGAATTG BRCA2 TG2-R: GGTGCCTATGGCCTGAAATA	230	58-35
BRCA2 TG3-F in FAM: CCAAGATATGAAGGCCAAAAA BRCA2 TG3-R: TTGATGTAAAGGTGGGGCTA	240	58-35
BRCA2 TG4-F: AGGTGATCCACTTGCCTT BRCA2 TG4-R in FAM: CACCTAACTTCTGTGACAAACAAT	225	58-35
BRCA2 AT1-Fin FAM: GCCTGGAAGCTACGAAGATG BRCA2 AT1-R: CCCCAAATATCATTACCGTGA	185	58-35
BRCA2 171-F in FAM: GCTTGCTCTGAGCTTCTGCT BRCA2 171-R: GAAGGAGAAAGGGGAGGTGT	180	58-35

PCR program:

Step1 **95** ºC for 2 min.

Step2 **94** ºC for 30 sec.

Step3 **58** ºC for 30 sec.

Step4 **72** ºC for 30 sec.

Step5 **go to step2, 35 cycles**

Step6 **72** ºC for 30 min.

Step7 **12** ºC forever.

2. **Dilution of PCR product with D.D water, 1:40.**

3.6.2 Genotyping Protocol

	1X
Hi-Di-Formamide (P/N: 4311320, L/N: 1002928)**:**	10.7µl
Rox (P/N: 402985 L/N: 1005364)**:**	0.3µl
PCR product (diluted)**:**	1.0µl

3. **Running on ABI 3130 Genetic Analyzer (S/N: 20355-023).**
4. **then results were analyzed using Gen-Mapper software.**

CHAPTER 4

Results

4.1 Sequencing Results

Sequencing of sixteen samples revealed two novel heterozygous nonsense mutations, E203X and E2229X in BRCA1 and BRCA2 respectively, in addition to frame-shift mutation 2482delGACT in BRCA2, which had been detected in 2002 with Palestinian breast cancer patient in Saudi Arabia [53].

4.1.1 E203X mutation

BRCA1 nonsense mutation, E203X is a heterozygous G to T mutation in exon 10. The mutation is presented in figure 4.

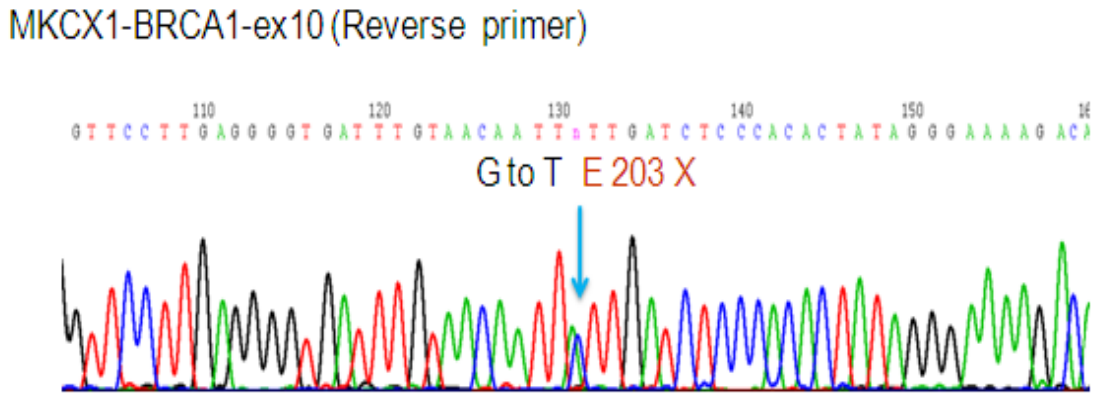


Figure 4: Identification of the novel E203X mutation, detected in a breast cancer patient in the heterozygous state.

It was found in a patient diagnosed with Breast cancer at age 42. The family pedigree is presented in figure 5.

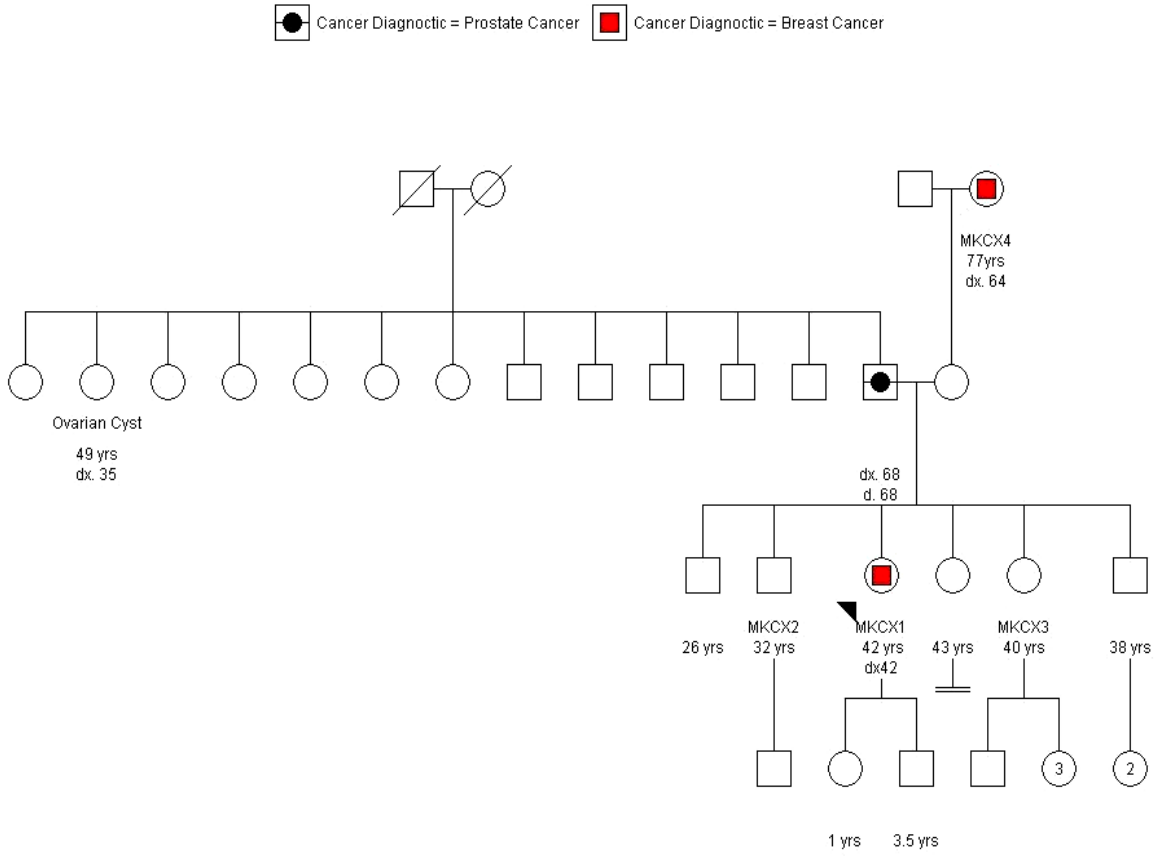


Figure 5: Family pedigree of patient, with whom E 203 X mutation was detected

4.1.2 E2229X mutation

E 2229 X- BRCA2 mutation is a heterozygous G to T mutation in exon11. The mutation is presented in figure 6.

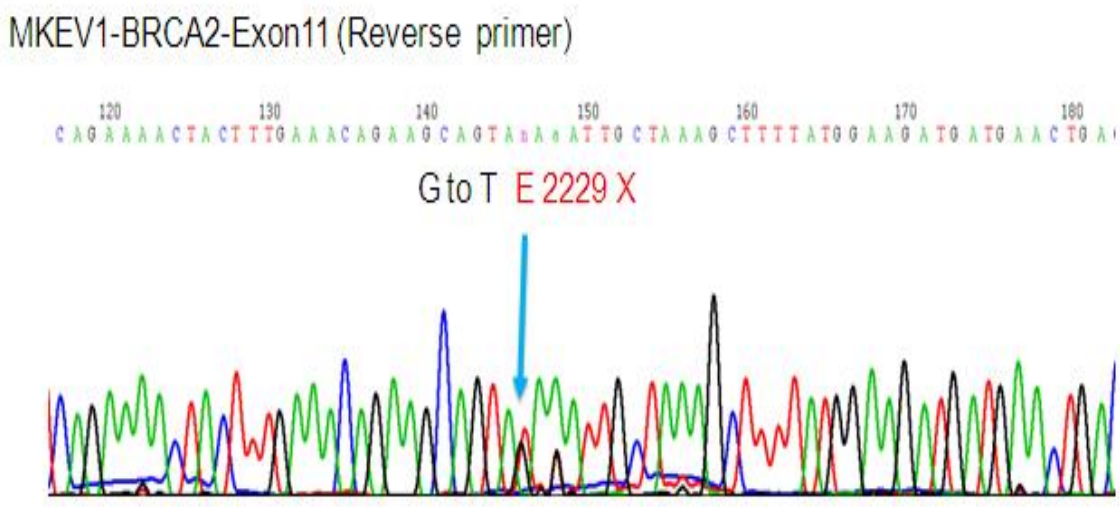


Figure 6: Identification of the novel E 2229 X mutation, detected in a breast cancer patient, in the heterozygous state. A - adenine, G -guanine, T - thymine, C - cytosine.

It was found in a patient diagnosed with Breast cancer at age 58. The family pedigree is presented in figure 7.

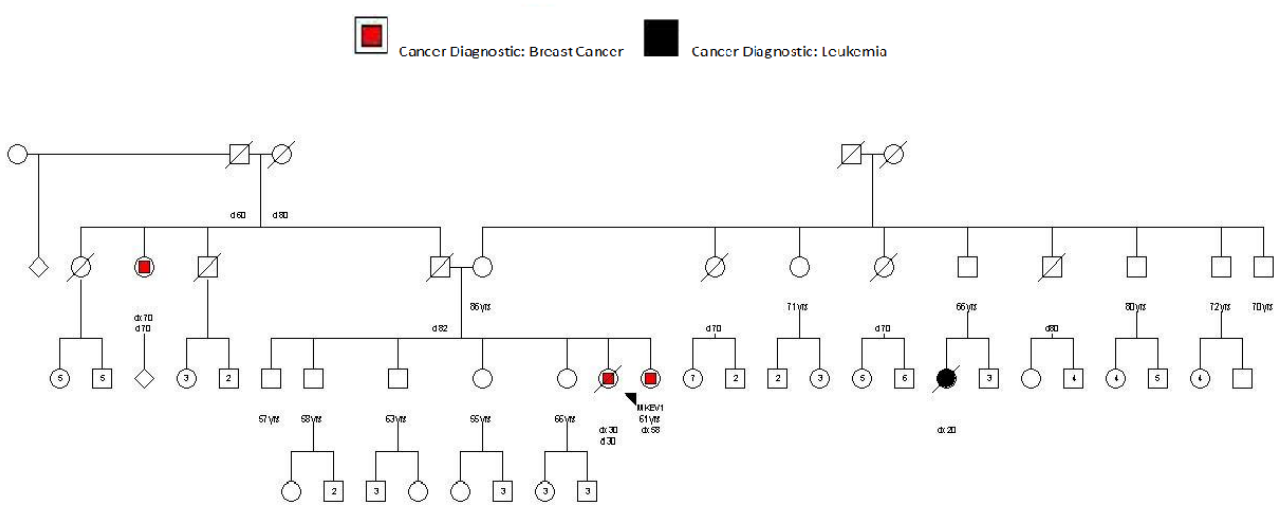


Figure 7: Family pedigree of the Breast Cancer patient, with whom E 2229 X mutation was detected

4.1.3 2482delGACT mutation

2482delGACT-BRCA2 mutation is a heterozygous frame-shift mutation; this mutation is a 4-nucleotide deletion that creates a stop signal at codon 770 of the BRCA2 transcript. The mutation is presented in figure 8.

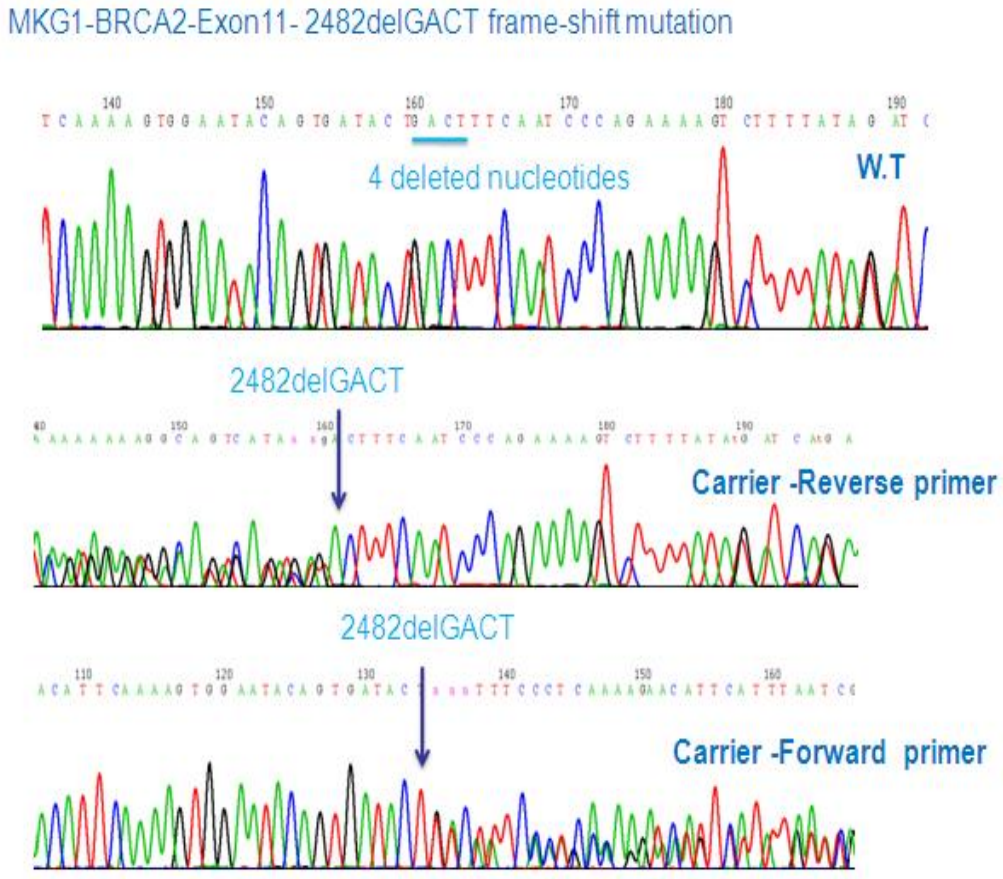


Figure 8: Identification of the frame-shift 2482delGACT mutation, a 4nucleotide deletion in exon 11 of the BRCA2 gene, detected in a breast cancer patient in the heterozygous state. An arrow marks the start of the deletion.

It was found in a patient diagnosed with Breast cancer at age 22. The family pedigree is presented in figure 9.

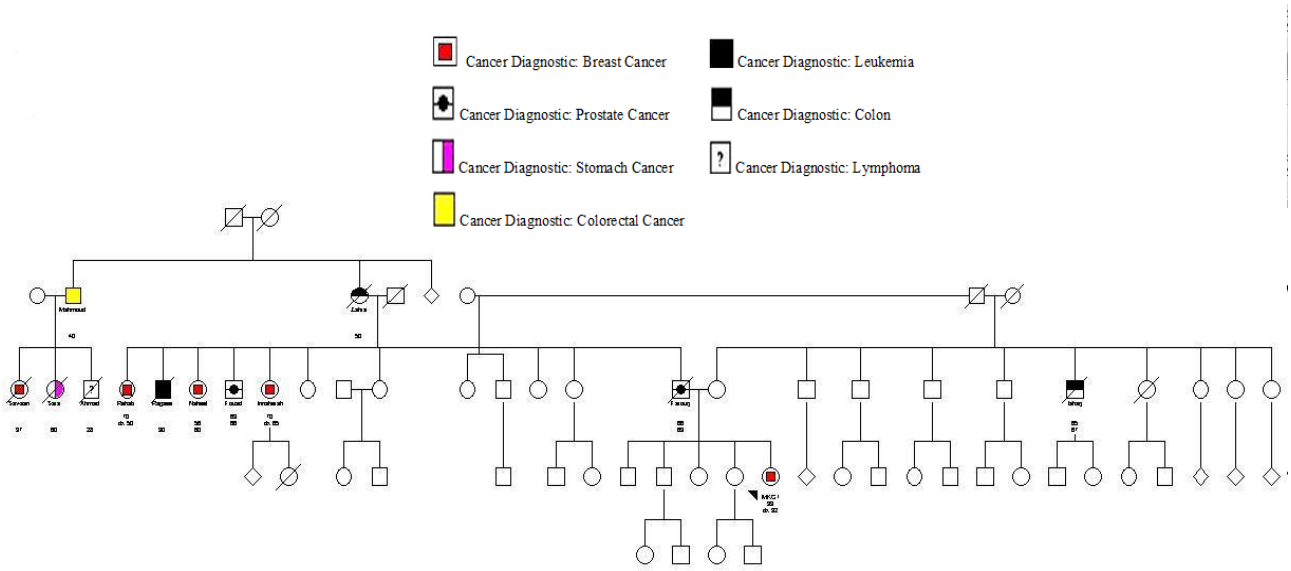


Figure 9: Family pedigree of the Breast Cancer patient, with whom frame-shift 2482delGACT mutation was detected

4.2 Screening Result

Screening of 364 patients for the E203X -BRCA1 mutation, using sequencing approach revealed no carriers of this mutation. Screening 364 patients for the E2229X- BRCA2 by enzymatic digestion using BfaI enzyme revealed two carriers of this mutation. Those two patients were diagnosed with Breast cancer are at age 50 and 47. The family pedigrees of them are presented in figure 11 and figure 12.

Sequencing of those two carriers confirmed digestion results. Illustration of digestion products is presented in figure 10.

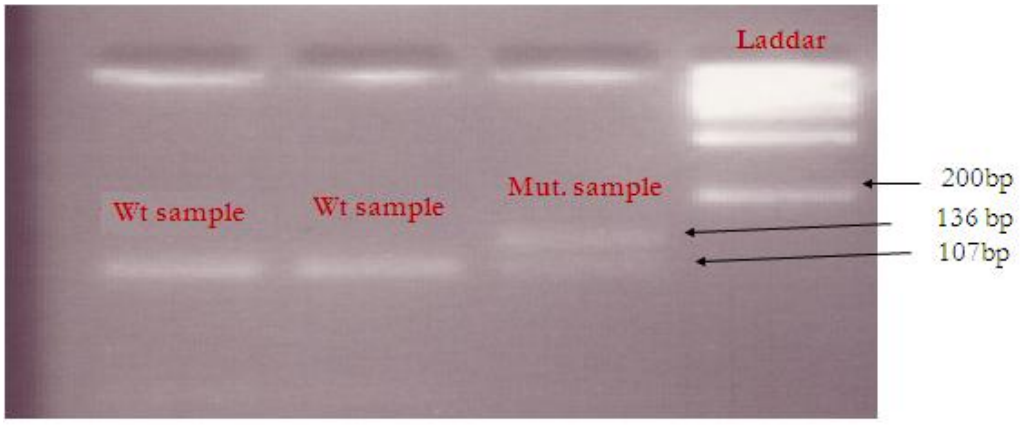


Figure 10: PCR digestion products for E2229X carrier and wild type sample.

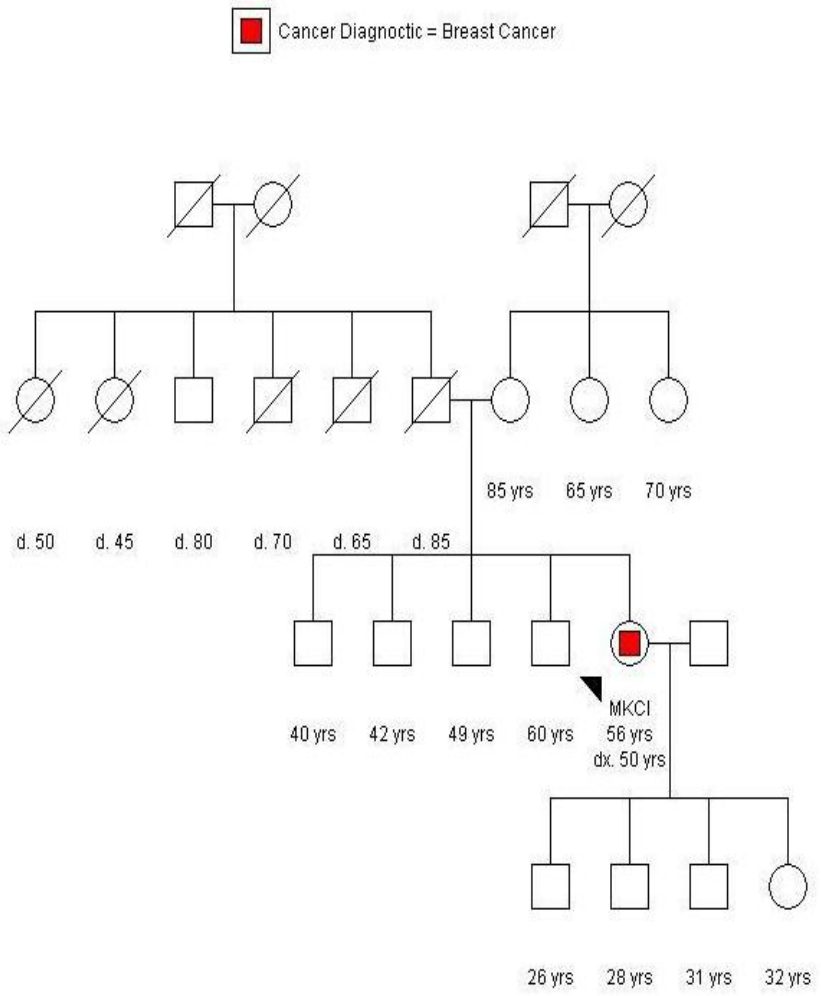


Figure 11: Family pedigree of the Breast Cancer patient, carrier for E 2229 X mutation

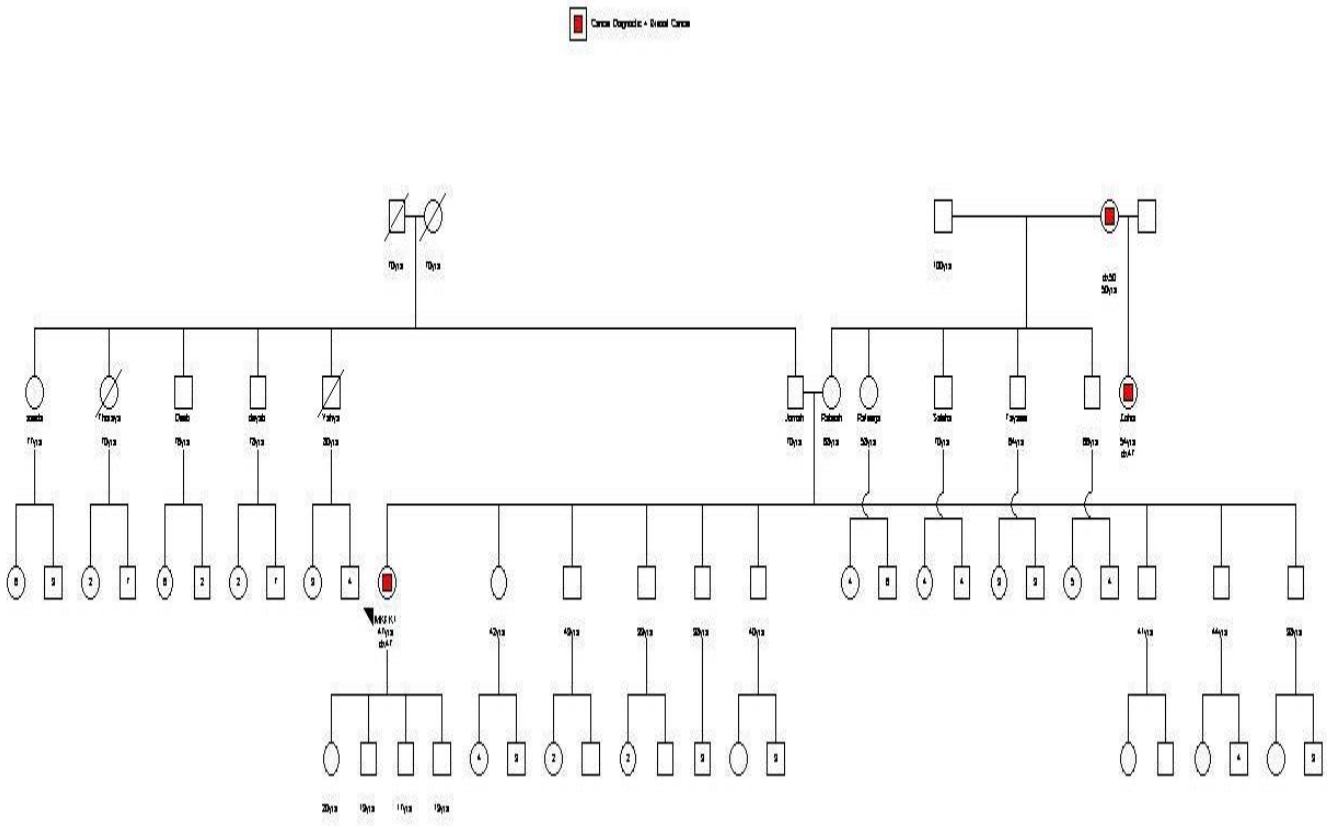
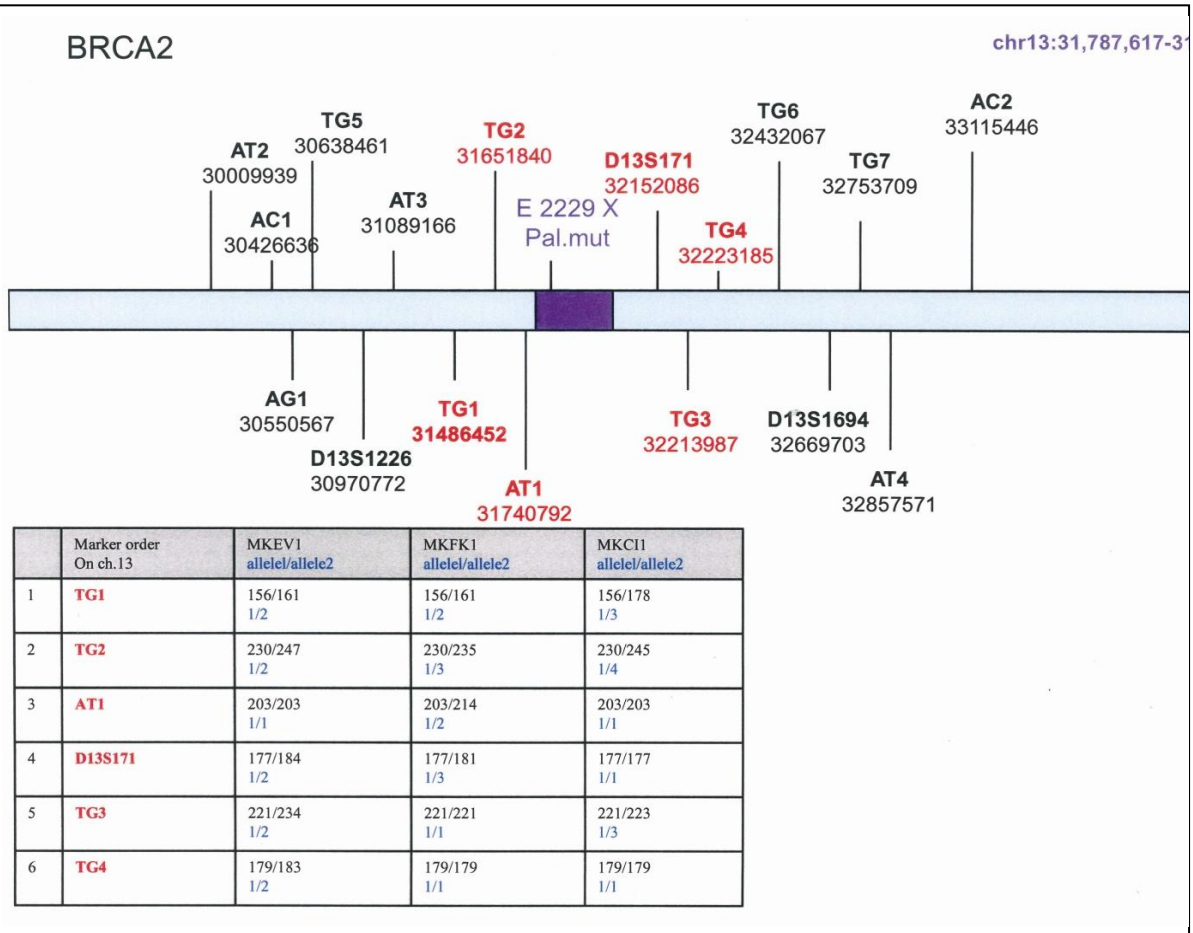


Figure 12: Family pedigree of the Breast Cancer patient, carrier for E 2229 X mutation

4.3 Haplotyping Results

Haplotyping for the six flanking microsatellite markers of BRCA2-exon-11, showed one homozygous haplotype block in three families affected with breast cancer and carrying the E2229X- BRCA2 mutation (figure 13), revealing that this shared mutation has a common haplotype founder effect.



	Marker order On ch.13	MKEV1 allele1/allele2	MKFK1 allele1/allele2	MKCI1 allele1/allele2
1	TG1	156/161 1/2	156/161 1/2	156/178 1/3
2	TG2	230/247 1/2	230/235 1/3	230/245 1/4
3	AT1	203/203 1/1	203/214 1/2	203/203 1/1
4	D13S171	177/184 1/2	177/181 1/3	177/177 1/1
5	TG3	221/234 1/2	221/221 1/1	221/223 1/3
6	TG4	179/183 1/2	179/179 1/1	179/179 1/1

Figure 13: Six flankig microsatellite markers around E2229X- BRCA2 mutation. Homozygous haplotype block was detected in three families affected with breast cancer and carrying the E 2229 X- BRCA2 mutation

4.4 Genotyping Results

Genotyping analysis for 365 samples revealed two carriers for 2482delGACT mutation, showing two peaks, a wild type at 251bp and a mutant, at 247bp. Normal control result is presented in figure 14, Carrier result is presented in figure 15, and pedigrees in figure 16 and 17.

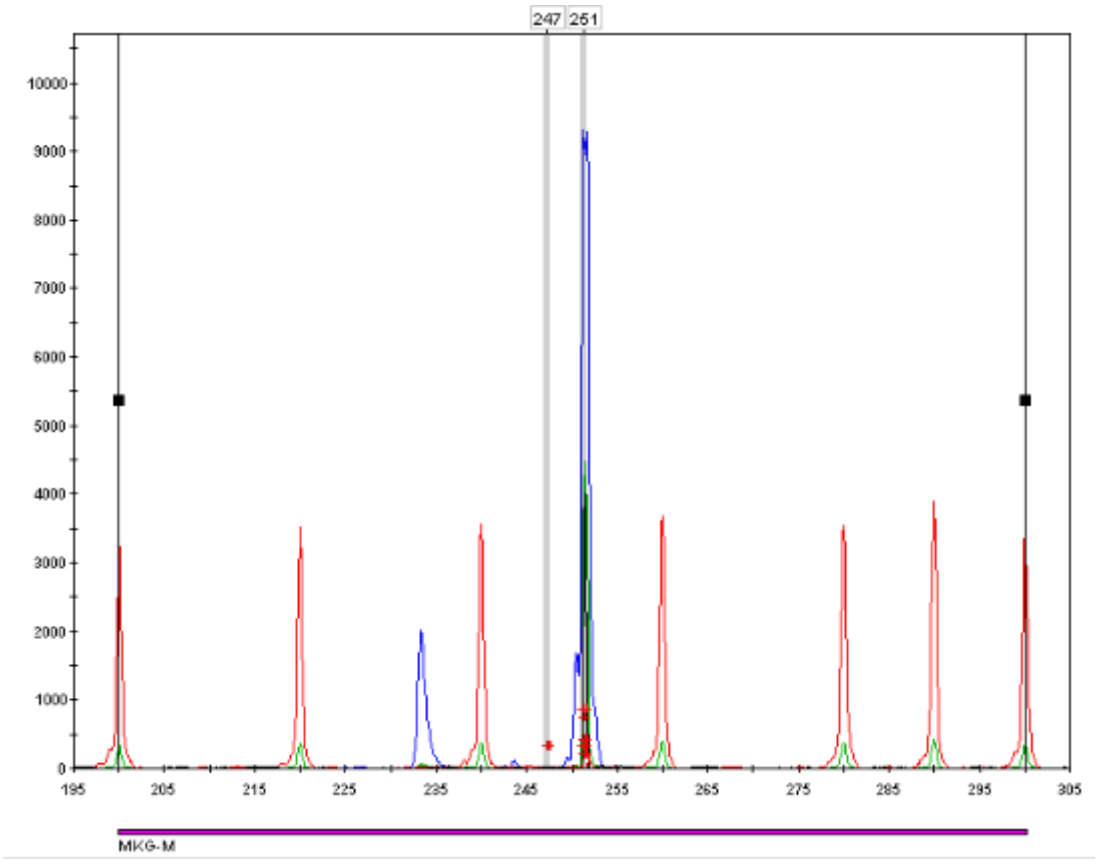


Figure 14: Control result for 2482delGACT-BRCA2 mutation, there is no deletion, only one peak is seen at 251 bp.

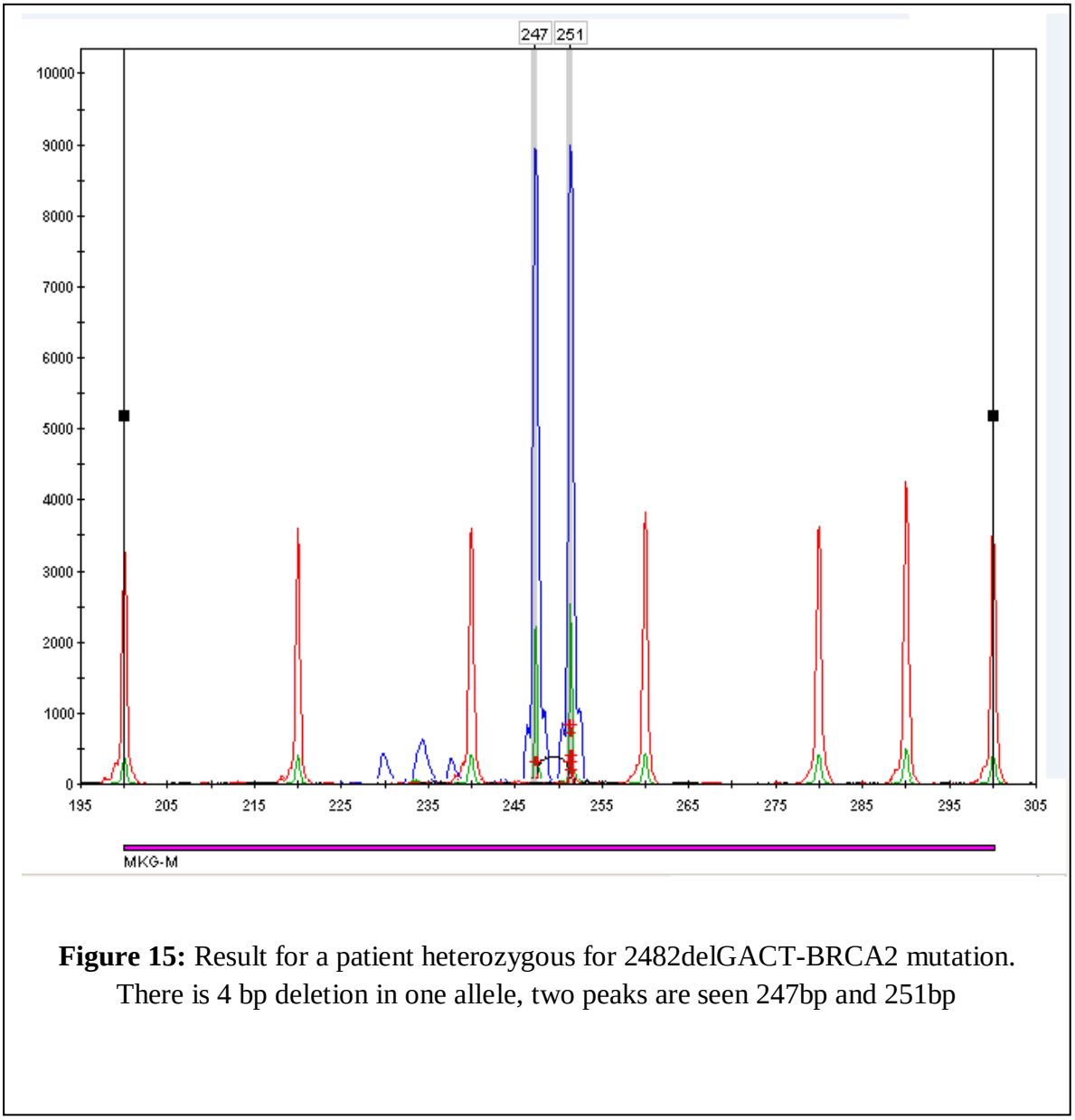


Figure 15: Result for a patient heterozygous for 2482delGACT-BRCA2 mutation. There is 4 bp deletion in one allele, two peaks are seen 247bp and 251bp

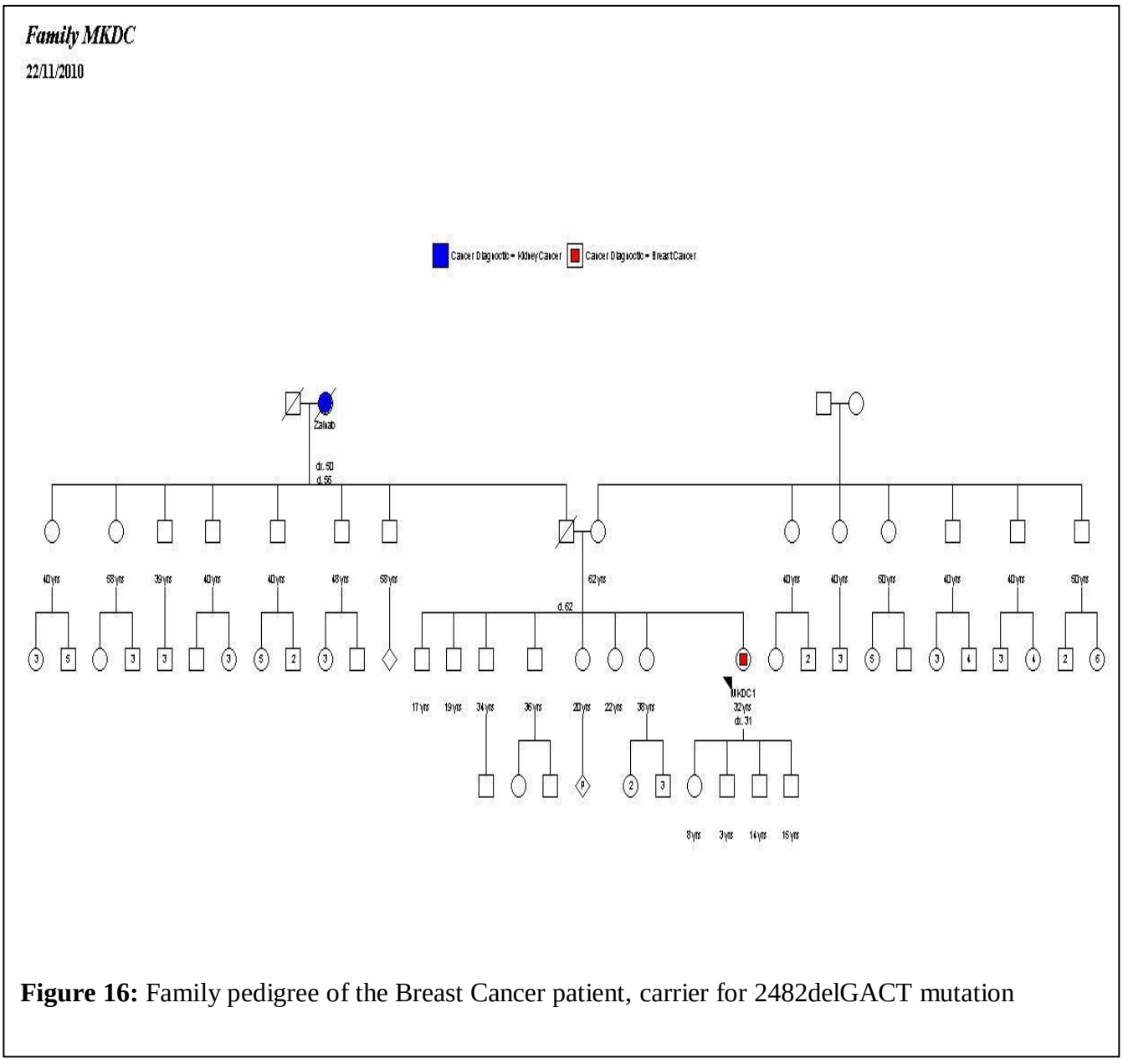


Figure 16: Family pedigree of the Breast Cancer patient, carrier for 2482delGACT mutation

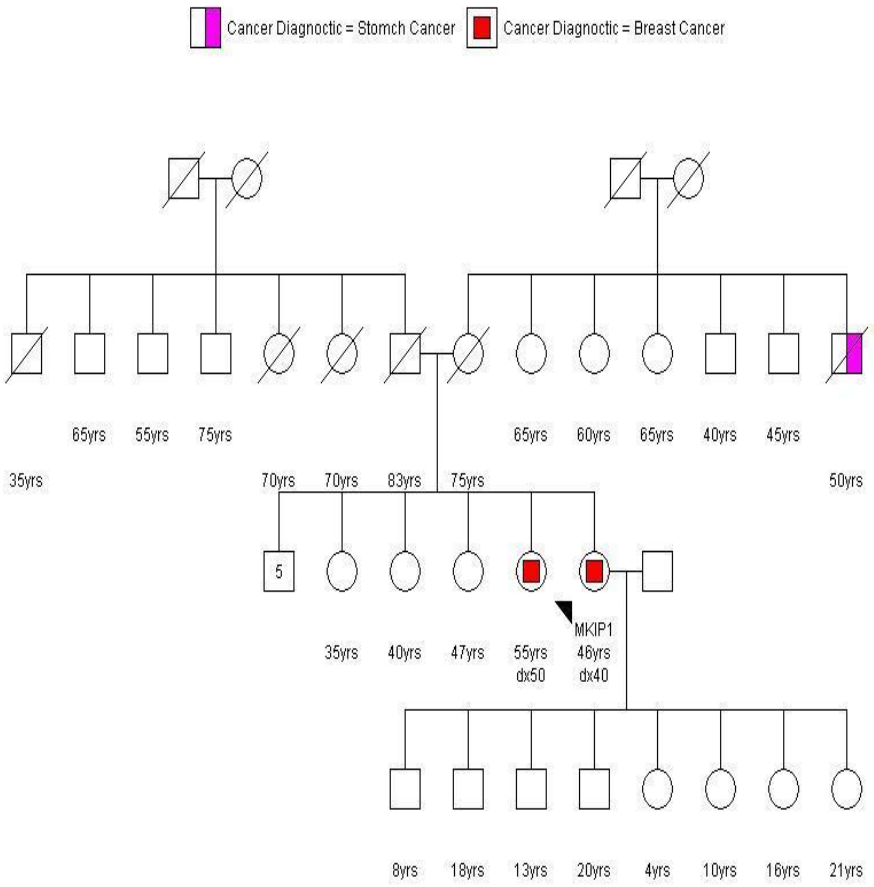


Figure 17: Family pedigree of the Breast Cancer patient, carrier for 2482delGACT mutation

CHAPTER 5

Discussion

In this study, we focus on identifying of BRCA1 and BRCA2 mutations among Palestinian women affected with Breast Cancer. Probands were selected based on a family history of breast and/or ovarian cancers. Sixteen probands were sequenced for BRCA1 and BRCA2 mutation detection. We have detected two novel heterozygous nonsense mutations, E203X and E2229X in BRCA1 and BRCA2 respectively, in addition to identified frame-shift mutation 2482delGACT in BRCA2, which had been detected in 2002 with Palestinian breast cancer patient from Saudi Arabia [53].

The E**203X** mutation changes G to T at base 4972222, resulting of a stop codon at codon 203. The mutation causes protein truncation and thus abolishes BRCA1 function. The affected woman was diagnosed with breast cancer at age 42; she has a family history with breast and ovarian cancers in her grandmother and ovarian cancer in her mother's sister. We tested DNA from her grandmother, brother, and sister and could not detect the mutation. Unfortunately we could not take DNA samples from her father's family. Haplotype analysis of informative family members is one approach to reveal whether this mutation was de novo or not. **E203X** mutation was not detected in 364 breast cancer women tested subsequently.

The **E2229X** mutation we identified in BRCA2 is G to T base change located at base 13895177 which creates a stop codon at codon 2229. The family history of this proband strongly suggest an inherited breast cancer risk. The sister of the affected woman was diagnosed with breast cancer at age 30 and the proband's grandmother had breast cancer. We have subsequently identified this mutation in two patients from our 364 cohort (Figures 11 and 12.). In both cases, a young onset of breast cancer was observed.

Haplotype analysis using six short tandem repeats markers (microsatellite markers), flanking the mutation revealed that the three affected probands shared the same haplotype for one of the two alleles. These results indicate that E2229X is a founder-effect mutation in Palestinian populations.

The **2482delGACT** is a frame-shift mutation that causes truncation of BRCA2 function. It was first detected in 2002 in a Palestinian breast cancer patient in Saudi Arabia [53]. Palestinian families with this mutation may have the same origin (ancestor). This mutation may be founder mutation in Palestinian population we found this mutation in a two patients with very young onset at ages 31 and 41 respectively (Figures 16 and 17).

5.1 Study significance

These elementary results reveal that risk for hereditary breast cancer in Palestine can occur by BRCA1 and 2 mutations but of a different nature than other populations. We do expect more population specific mutations to be revealed further study. We also believe that our population-specific profile will be instrumental in setting the genetic basis for breast cancer risk assessment, testing and management in this population. The awareness

created by this work will also provide a model whereby genetic services and counseling become etiologically based.

Conclusion

We conclude that there is a significant contribution of BRCA1 and BRCA2 mutations in the pathogenesis of familial breast cancer in Palestine. Based on haplotyping analysis of (E2229X) mutation, novel G to T (E2229X) mutation is a founder mutation in the Palestinian population. We tried to define the genetic component of breast cancer incidence among the Palestinian population. However, only full sequencing of the BRCA1and BRCA2 genes may provide complete picture regarding the role of BRCA1 and BRCA2 mutations in the incident of breast cancer in the Palestinian population. Based on our study full sequencing of BRCA1 and BRCA2 should be done for patients with characteristic family history for breast cancer.

Appendix A

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

Primer Name	Sequence
B2.2.F	TTCCAGGAGATGGGACTGAATTAG
B2.2.R	AAGCAACACTGTGACGTACTGGG
B2.3.F	GCCATCTTGTAACTTTTGTGAACTC
B2.3.R.outer	CGTAATTCAGCAAATCTCAGTTGG
B2.3.R.seq	GTCTCCATTTTTCGAGTGAGG
B2.4.F	GTAATCAGCAAACTGAAAAACC
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	CCACATCCTAGTGGTGCAAGATTTC
B2.9.R	CTACTTGGCAAGCTGAGGCAGG
B2.10A.F	GCTTTACTAGAAGAACAGGAGAAGG
B2.10A.R	GGATCAGTATCATTTGGTTCCAC
B2.10B.F	GATACCTCTGAAGAAGATAGTT
B2.10B.R	GTATGAGATTCAAGATGCTG
B2.10C.F	GCATATTTCTTCATGTGACCA
B2.10C.R	GCCAGCTTCCATTATCAATTA
B2.10D.F	TTAAAAAAGAAACTGAAGCCTC
B2.10D.R.outer	GTGACCTGATTCTAAACACTGG
B2.10D.R.seq	AAAAAAGACAGAGGTACCTGAATC
B2.11A.F	CCCAAAAGTGCTGAGATTACAG
B2.11A.R	GAGTTAAAATAAGAGTGCTGGC
B2.11B.F	GGACAGTGTGAAAATGATCC
B2.11B.R	TGAGAAAAGTTCTTCAGAGTCTG
B2.11C.F	CCACCTGAAAAATACATGAGAG
B2.11C.R	GTGATTTGAAATTGGACCTAAG
B2.11D.F	ACCCAAGTGTCAATTAAAAAAG
B2.11D.R	GTTTGAATTAAAATCCTGCTTG
B2.11E.F	CAAAAGAAACTGAGCAAGCC
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	AAAACAGAACAAAAATGTAATTG
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	TTTGTTTTCACTTTTAGATATGATAC
B2.18B.R	TGTCTGAAGAATATGCTTTTTATAC
B2.19.F	CATTAAGAACTTGTAGCAGTATAAAC
B2.19.R.outer	TGCGAAATATGTATAATCCAG
B2.19.R.seq	AAGATCACGCCATTGCACTC
B2.20.F.outer	TATCATCGCTTTTCAGTGATGG
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	CATTCCCCCATCTCCTGAGG
B2.26.F	CTCTCCCTATCAGCTAGATTCCCC
B2.26R	CTTACAGGAGCCACATAACAACCAC
B2.27A.F	ACCTATTAGGAGTTAGGGGAGG
B2.27A.R	GAACCAGACAAAAGAGCTT
B2.27B.F	CATTTCAGCCACCAAGGAGTTG
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	CAAACTGCACATACATCCCTGAAC
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	GCAGCATTCAGAAAGTTAATGAGTG
B1.11C.R	TCCGTTTGGTTAGTTCCCTG
B1.11D.F	CTGAATCAAATGCCAAAGTAGC
B1.11D.R	TGGGTTAGGATTTTTCTCATTC
B1.11E.F	ATATCCACAATTCAAAAGCACC
B1.11E.R	CAAAACCCTTTCTCCACTTAAC

B1.11F.F	TGCACCTGGTTCTTTTACTAAG
B1.11F.R	TCTTCTGCATTTCCTGGATTTG
B1.11G.F	GAAGGCTTTAAGTATCCATTGG
B1.11G.R	GACTTGATGGGAAAAAGTGGTG
B1.11H.F	GCCAGTTGATAATGCCAAATGTAG
B1.11H.R	GGATGCTTACAATTACTTCCAGGA
B1.11I.F	AACATTCAAGCAGAACTAGG
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	AAAACTCTTTCCAGAATGTTGT
B1.17.F.outer	GCCGAGACTTCAGGTGTCTTAG
B1.17.R.outer	GGAAGCAGCAAAACTATTATTTGTC
B1.17.F.seq	AAATAGTTCCAGGACACG
B1.17.R.seq	TCCTAATCTCGTGATCTGCCCG
B1.18.F	GGCTCTTTAGCTTCTTAGGAC
B1.18.R	GAGACCCATTTTCCCAGCATC
B1.19.F	CTGTCATTCTTCCTGTGCTC
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

Appendix B

PCR programs for all the primers, used in amplification of BRCA1 and BRCA2

BRCA1				
Primers	U14680 region	Size	PCR cond.	DMSO
1		531	TD60	+
2		344	TD50	+
3		427	TD50	+
5		482	TD50	+
6		415	TD50	+
7		349		
new7R			TD55	+
8		291	TD50	+
9		306	TD50	+
10		281	TD50	+
11A	intron-1090	401	TD50	+
11B	944-1375	432	TD50	+
11C	1249-1738	490	TD50	+
11D	1327-1829	503	TD50	+
11DD	1659-2073	415	TD50	+
11E	1915-2393	479	TD50	+
11F	2231-2758	528	TD50	+
11G	2598-3082	485	TD50	+
11H	2930-3436	507	TD60	+
11I	3318-3789	472	TD50	+
11J	3612-4130	519	TD50	+
11K	3952-intron	418	TD50	+
12		265	TD50	+
13		499	TD50	+
14		312	TD50	+
15		509	TD50	+
16		450	TD50	+
17		**844 outer**	TD50	+
18		352	TD50	+
19		249	TD50	+
20		401	TD50	+

21		416	TD50	+
22		399	TD50	+
23		**682 outer**		
23a				
23a2F+23aR			TD50	+
24		280	TD50	+

BRCA2				
Primers	U43746 region	Size	PCR cond.	new PCR cond
2		321	TD45	+
3		**721 outer**	TD50	-DMSO
4		476	TD50	-DMSO
5,6		520	TD45	-DMSO
7		488		
7new			TD55	-DMSO
8		452	TD45	-DMSO
9		491	TD60	-DMSO
10A	intron-1322	496		
10Anew			TD55	-DMSO
10B	1129-1661	533	TD45	+
10C	1470-1918	449	TD45	+
10D	1814-intron	**495 outer**	TD45	-DMSO
11A	intron-2545	536	TD60	-DMSO
11B	2371-2880	510	TD45	+
11C	2731-3237	507	TD45	+
11D	3043-3528	486	TD45	+
11E	3394-3904	511	TD45	+
11F	3691-4210	520	TD45	-DMSO
11G	3985-4553	569	TD45	-DMSO
11H	4418-4963	546	TD45	+
11I	4763-5291	529	TD55	-DMSO
11J	5165-5676	512	TD60	-DMSO
11K	5544-6048	505	TD45	-DMSO
11L	5888-6409	522	TD45	+

11M	6213-6595	383	TD45	+
11N	6399-6890	492	TD45	+
11O	6769-intron	449	TD55	+
12		432	TD50	-DMSO
13		507	TD50	-DMSO
14A	intron-7623	539	TD45	-DMSO
14B	7409-intron	490	TD45	+
15		473	TD60	-DMSO
16		583	TD60	-DMSO
17		500	TD45	-DMSO
18A	intron-8436	464	TD45/ td55?	-DMSO ???
18B	8203-intron	520	TD45	-DMSO
19		**825 outer**	TD45	-DMSO
20		**794 outer**	TD45	-DMSO
21		390	TD45	+
22		497	TD55	+
23		507	TD45	+
24		419	TD45	+
25		572	TD45	-DMSO
26		491	TD45	+
27A	intron-10295	554	TD45	-DMSO
27B	10118-stop	520	TD60	-DMSO

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